TARGETED DISRUPTION OF THE INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3 GENE IN MOUSE EMBRYONIC STEM CELLS

By

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the requirements
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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
kb	kilobase
^{0}C	degree centigrade
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CTP	cytosine triphosphate
dNTP	deoxyribonucleotide triphosphate
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ES cell	embryonic stem cell
FBS	fetal bovine serum
G418	geneticin
hr	hour (s)
HEPES	N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
HSV-tk	herpes simplex virus thymidine kinase
IGF	insulin-like growth factor
IGFBP-	insulin-like growth factor binding protein
kDa	kilodalton
L	litre

LIF	leukemia inhibitory factor
min	minute
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
M	molar
Neo^r	neomycin resistance gene
ng	nanogram
nM	nanomolar
O/N	overnight
PCR	polymerase chain reaction
pg	picogram
PBS	phosphate buffered saline
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
TE	Tris-HCl/ EDTA
μg	microgram
μl	microlitre

ABSTRACT

Insulin-like growth factors (IGFs) play important functions in cell proliferation, differentiation and metabolism. The biological actions of IGFs are mediated through binding to the specific receptor-IGF-I receptor. However, in the circulation IGFs are associated with their binding proteins, the insulin-like growth factor binding proteins (IGFBPs). Among the six IGFBPs, IGFBP-3 is the predominant binding protein for IGFs in circulation. IGFBP-3 is believed to play important regulatory roles in the biological actions of IGFs. Although many functions of IGFBP-3 have been described in the last decade, the results were not consistent. The physiological function of IGFBP-3 has not yet been defined. To study the physiological function of IGFBP-3, gene targeting technique was employed to generate a null IGFBP-3 mouse model. The mouse IGFBP-3 gene was obtained by screening a mouse genomic library using a full length human IGFBP-3 cDNA. The mouse IGFBP-3 gene restriction map was established by restriction analysis of the mouse IGFBP-3 gene by using different fragments of human IGFBP-3 cDNA as probes. A gene targeting vector for the mouse IGFBP-3 was successfully constructed by inserting two fragments from mouse IGFBP-3 gene into pPNT vector in which Neo^r and HSV-tk genes have already been engineered. An average of 422 colonies were obtained from $1.5x10^7$ ES cells which survived elctroporation. Of 550 ES cell colonies screened by Southren blot hybrization, two ES cell colones were shown to carry homologous recombination for mouse IGFBP-3 locus. The targeting frequency is 0.36%. Seven chimeric mice were generated by blastocyst injection, none of them has been

found to carry the germline transmission. Successful deletion of mouse IGFBP-3 gene in ES cell and generation of chimeric mice for IGFBP-3 have been achieved in this study. More chimeric mice will have to be developed to obtain germline transmission.

Chapter 1. LITERATURE REVIEW OF IGFBP-3

1.1 Introduction

Insulin-like growth factors (IGF-I and IGF-II) are peptides with significant structural homology to insulin (Rinderknecht et al., 1978), but unlike insulin, which is mainly produced in pancreas, IGFs are synthesized and secreted in many tissues and have important functions in regulating cell growth, differentiation and metabolism. In mice, IGF-II is expressed highly in the fetal period and decreases after birth; whereas IGF-I is expressed at relatively low level during fetal period and increases after birth. The biological action of IGFs is mediated through binding to their specific receptors (type I receptor) on the cell surface with high affinity. However in the circulation insulin-like growth factors (IGF-I and IGF-II) are associated with insulin-like growth factor binding proteins (IGFBPs). IGFBPs are a family of at least six different proteins (designated IGFBP-1 - IGFBP-6) which have been cloned and characterized from different species (Shimasaki et al., 1991; Baxter et al., 1989; Jones et al., 1995). IGFBP-7 has recently been identified which shares 20-25% homology to human IGFBPs (Oh et al., 1996). IGFBPs share about 35% sequences identity with each other, and they are present in a variety of tissues. IGFBPs are served to either inhibit or potentiate the action of IGFs (Baxter et al., 1989; Jones et al., 1995) depending upon the experimental conditions. They may also provide a storage pool and prolong the biological half-life of IGFs in circulation (Cohick et al., 1993). The general characteristics of the six known IGFBPs

are summarized in the table1.

Table 1. General Characteristics of the human IGFBPs

	No. of amino acids	Core molecular mass	Special features	IGF affinity	Modulation of IGF action
IGFBP-1	234	25.3	REG	I=II	inhibition and/or potentiation
IGFBP-2	289	31.4	REG	II>I	inhibition
IGFBP-3	264	28.7	N glycosylation	I=II	inhibition and/or potentiation
IGFBP-4	237	25.9	2 extra cysteine	I=II	inhibition
IGFBP-5	252	28.5	extracellular matrix hydroxyapatite binding	II>I	potentiation
IGFBP-6	216	22.8	2 less cysteine O-glycosylation	II>I	inhibition

RGD, Arg-Gly-Asp

Although the IGFBPs differ in their stucture and binding specificity, it is not clear whether these differences contribute to the functional differences among the various IGFBPs. Studies showed that different IGFBPs modulate IGF action through different ways. However, the precise role of six diffeent IGFBPs is still poorly understood (Clemmons, 1992). Among the six IGFBPs, IGFBP-3 is the predominant binding protein

of IGFs in circulation. About 75%-80% of IGFs are bound to IGFBP-3 together with an 85 kDa acid-labile subunit forming a ternary complex in circulation (Rechler, 1993). Many studies have suggested the IGFBP-3 plays important roles in the regulation of the biological action of IGFs, but the results are inconclusive. The following literature review details the studies on IGFBP-3 so far undertaken.

1.2 Molecular characterization of IGFBP-3

The IGFBP-3 was first isolated in human serum and the description of its N-terminal amino acid sequence was reported in 1986 by Baxter et al (Baxter., at al 1986). Subsequently the IGFBP-3 from the rat, mouse, pig and cattle were isolated and N-terminal sequences reported by different groups (Walton et al.,1989; Blat at al., 1989; Conover et al., 1990; Walton et al., 1990). Complementary DNA clones of IGFBP-3 were isolated from human (Wood et al., 1998; Spratt et al., 1990), rat (Albiston and Herinton., 1990), porcine (Shimasaski et al., 1990) and bovine (Spratt et al., 1991) liver and ovarian cDNA libraries (Shimasaki et al., 1989). The numbers of amino acids for human, bovine, porcine, and rat IGFBP-3 are 264, 264, 266, and 265 respectively. The deduced amino acid sequences of the IGFBP-3 from these four species are quite similar: 64% in exon 1, 68% in exon 2, 95% in exon 3, and 80% in exon 4. Residues 155 - 247 (extending from exon 2 to exon 4) are identical in rat and human IGFBP-3; porcine and bovine IGFBP-3 are identical to each other in this region and differ from human and rat IGFBP-3 by only two conservative substitutions. The most homologous region in the four

IGFBP-3 is the C-terminal portion of the molecule. The locations of all 18 cysteines in these IGFBP-3s are conserved. Smaller fragments of IGFBP-3 were also isolated from human and rat serum (Shimonaka et al., 1989; Shimasaki et al., 1991; Sommer et al.,1991; Zapf et al ., 1990). They are classified into 2 groups, a C-terminal truncated group and N-terminal truncated group. Both groups of smaller fragments of IGFBP-3 may be generated post-translationally by protein cleavage. Since they were isolated by using IGF-affinity columns, they retain the ability for binding IGF although their binding affinity was reported to be low (Giudice et al., 1990; Hosenlopp et al., 1990).

1.3 IGFBP-3 gene structure and promoter

The human IGFBP-3 gene is a single copy gene and its locus was mapped to chromosome 7 (Cubbage et al., 1990; Shimasiki et al., 1991). It spans 8.9 kb and consists of 4 introns and 5 exons in contrast to the 3 introns and 4 exons in IGFBP-1 and -2 genes (Brinkman et al., 1988; Ehrenborg et al ., 1991). Exon 1 of the human IGFBP-3 gene includes the 5' noncoding region and coding region for the signal sequence and first 80 amino acid sequence, which contains 12 conserved cysteines. Exon 2 spans 227bp, which encodes 76 amino acids. Exon 3 has 120 bp, which encodes 40 amino acids. Exon 4 has 141bp which encodes C-terminal 41 amino acids, the stop codon and 15 bp of 3' non-coding sequence. Exon 5 contains only the 3' non-coding region. Intron 1 is the largest IGFBP-3 intron, at 3285 bp, while the 544-bp intron 2 is the smallest; intron 3 and 4 are 1595 and 941 bp respectively.

The major cap site was identified by primer extension at 132 bp 5' to the initiation methionine codon. This is consistent with an IGFBP-3 mRNA transcript 2.5kb in length. A consensus TATA box sequence and a GC upstream promoter element were found at the adjacent nucleotide region upstream of the cap site. The cis-acting elements responsible for regulating the IGFBP-3 gene transcription have not yet been determined.

1.4 Biological Action

1.4.1 Inhibition of IGF action

DeMellow and Baxter found that purified IGFBP-3 added with IGF-I to human skin fibroblasts significantly inhibited IGF-I-stimulated DNA synthesis (DeMellow and Baxter, 1988). Maximal inhibition was achieved at the concentration of 80 ng/ml of IGFBP-3 in the presence of 7.5ng/ml of IGF-I. Blat and colleagues found that a form of IGFBP-3 purified from the mouse has consistently inhibited IGF action in several bioassays (Blat at al., 1989). They observed that this IGFBP-3, when added in 3- to 4-fold molar excess, exerted an inhibitory effect on DNA synthesis by IGF-I in chick embryo fibroblasts. More recently, IGFBP-3 was shown to inhibit glucose incorporation in BALB/c3T3 cells (Okajima et al.,1993), but excess of IGFBP-3 was required to inhibit IGF-stimulated actions. However, IGFBP-3 had no effect on insulin-stimulated glucose incorporation. Preincubation of IGFBP-3 alone showed inhibition of subsequent effects of IGF-I or IGF-II.

The mechanism by which IGFBP-3 inhibits the action of IGFs is related to its ability to inhibit IGF binding to IGF-I receptor. Only the free form of IGF-I in circulation can bind to type I receptor, IGFs complexed with IGFBP-3 can not bind to the receptor. IGFBP-3 in solution has a significantly higher affinity for IGF-I and II than does the type I receptor, thus it is clearly capable of preventing receptor interaction. In contrast, when IGFBP-3 associates with cell surface, its affinity for IGF-I and II is reduced (McCusker et al., 1990), so it is capable of modulating IGF receptor interactions such that IGFs are more available for receptor binding (McCuske et al., 1991).

Some studies showed that IGFBP-3 inhibited IGF actions in differentiated cells. Biscsak et al found that IGFBP-3 could inhibit cAMP generation and DNA synthesis induced by IGF-I in rat granulosa cells (Bicsak., 1990). Schmid et al found that 10 nM intact IGFBP-3 was a potent inhibitor of the effect of 1nM IGF-I on DNA synthesis and collagen synthesis in cultured osteoblasts (Schmid., 1991). In this study, it was shown that only the intact but not truncated IGFBP-3 has the inhibitory effect on IGF action. Andress and Birnbaum obtained a similar result in mouse osteoblasts (Andress et al., 1992). One study showed that porcine IGFBP-3 inhibited the IGF-I-stimulated glycogenolysis and glucose oxidation in porcine fat cell, and this inhibitory effect could be overcome by increasing concentrations of IGF-I (Walton at al., 1989). However, porcine IGFBP-3 had no effect on insulin stimulation, suggesting the inhibitory mechnism of IGFBP-3 on IGF is probably the prevention of IGF binding to its receptor. Cohen et al transfected BALA/c 3T3 fibroblast with an IGFBP-3 cDNA, after expression of

recombinant protein, they found these cells had a longer doubling time than cells transfected with plasmid alone and the final cell density was lower (Cohen et al., 1993).

1.4.2 Potentiation of IGF actions

Cornel et al found that a serum fraction containing largely IGFBP-3 could potentiate the effects of IGF and glucose incorporation (Cornell et al., 1986). The studies carried out by DeMellow and Baxter (DeMellow and Baxter, 1988) showed that preincubation of human fibroblasts with IGFBP-3 followed by its removal resulted in substantial potentiation of the effects of IGF-I on DNA synthesis, whereas coincubation resulted in inhibition. The addition of 50ng/ml of IGFBP-3 for 4-48h before the addition of IGF-I resulted in a 2-fold stimulation of thymidine incorporation compared with IGF-I alone. Conover et al (Conover et al., 1990) observed that bovine IGFBP-3, when coincubated with increasing concentration of IGF-I, produced a dose-dependent inhibition in AIB uptake by cultured fibroblasts. In contrast, when the fibroblasts were preincubated with bovine IGFBP-3, which was then removed, there was dose-dependent enhancement of RIB uptake response to IGF-I with 32 to 86% increases detected after 24h of exposure to 10 nm IGFBP-3. Potentiation could be further enhanced by perincubating for 72h with IGFBP-3. Blum et al showed that the baby hamster kidney cells cocultured with IGF-I and IGFBP-3 had a 90% increase in DNA synthesis as compared with IGF-I alone (Blum at al., 1989). The effects occurred between 12 and 15h after the addition of the reagents.

The concentration of IGFBP-3 used was 180 ng/ml, and higher concentration resulted in inhibition of IGF-I action, suggesting that an optimal ratio of IGFBP-3 to IGF-I was required to produce a potentiating effect.

Proteolysis is considered to be the posttranslational processing mechanism that might affect the actions of IGFBP-3. It is now known that plasma contains a protease that specifically cleaves IGFBP-3 into a 30 kDa form that has a markedly reduced affinity for IGF-I. Lower affinity of IGFBP-3 has been shown to potentiate IGF actions. Schmid et al (Schmid at al., 1991) tested the effect of a purified 30 kDa proteolytic fragment of IGFBP-3 on thymidine corporation into DNA in a rat osteoblast cell line. The truncated IGFBP-3 significantly potentiated the effect of added IGF-I, whereas native IGFBP-3 was inhibitory. It was suggested that modulation of IGF action by IGFBP-3 depends upon entirety of the structure of IGFBP-3 or the binding capacity of IGFBP-3 to IGF which is determined by the structure of IGFBP-3. Truncated IGFBP-3 concentrations between 0.1 and 10 nm resulted in major potentiation of the growth of control cultures and a modest but significant potentiation of growth in response to IGF-I. This suggested that the IGFBP-3 fragment might have intrinsic mitogenic activity independent of IGF-I. Conover and Powell (Conover and Powell, 1991) suggested that the major mechanism mediating this may be prevention of receptor down-regulation.

Studies above suggested that cell surface association seems to be required for IGFBP-3 to potentiate IGF action. Since localization to the cell surface results in

significant reduction of the affinity of IGFBP-3 for the IGFs, this may be a major mechanism accounting for its effect (Jones and Clemmons, 1995).

1.4.3 Direct inhibition of cell growth

Villaudy et al (Villaudy et al., 1991) found that IGFBP-3 inhibited DNA synthesis in mouse embryo fibroblasts that had been stimulated by either 1% serum or FGF by about 65%. This study showed for the first time that IGFBP-3 could inhibit responsiveness to other growth factors and suggested that it might have inhibitory potential independent of IGF binding. However, the possibility that the FGF stimulated local synthesis of IGF that was bound by IGFBP-3 was not excluded. Oh et al have shown that IGFBP-3 can inhibit the growth of human breast cancer cells independently of IGF stimulation (Oh et al., 1993). Specifically they found that the addition of recombinant IGFBP-3 bound to these cells in a dose-dependent manner if calcium chloride and magnesium were included in the incubation medium, and they showed a dose-dependent (60%) inhibition of cell growth at 20nM of IGFBP-3. They also obtained a significant inhibition at 10 nM but had no effect with IGFBP-1. No IGF was added to this system although the possibility that the cells synthesized IGF-I or II was not rigorously excluded. The IGFBP-3 inhibitory effect could be partially overcome by coincubation with native IGF-I but not by coincubation with IGF analogs. These experiments suggest that IGFBP-3 has inhibitory effect that independent of its capacity to bind to IGF-I. Lalou and colleagues found that the two fragments of IGFBP-3 generated by proteolysis induced by

plasmin, one has weak affinity for IGF and the other lacks the affinity for IGF (Lalou at al.,1996).

1.4.4 In vivo studies

The Hunt Schilling wound healing model was applied to show that the use of equimolar concentrations of IGFBP-3 and IGF-I results in acceleration of wound healing and increased amount of wound tissue (Sommer et al., 1991), whereas IGF-I alone did not result in such potentiation. The study in an impaired wound healing model showed the same result, it also showed that combination of IGF-I plus IGFBP-3 had a greater effect on preventing catabolism. Clark et al (Clark et al., 1993) showed that the subcutaneous injection of human IGFBP-3 and IGF-I to GH deficient rats resulted in 2-fold more weight gain and significantly greater epiphyseal width compared with IGF-I alone.

1.4.5 Transgenic animal

Only one transgenic model of IGFBP-3 generated by pronuclear injection of an hIGFBP-3 cDNA driven by the mouse metallothionein 1 promoter has been reported by Murphy et al (1995) so far. In this model, immunoreactive hIGFBP-3 was detected in the 140 kDa ternary complex, but the majority was not associated with the acid-lable subunit. The transgenic mice were phenotypically normal at birth. However, selective

organomegaly was found. The spleen, liver and heart of transgenic mice were significantly heavier than those organs of wild type. Murphy et al suggested that this organomegaly may be the result of enhanced circulating levels of non-ternary IGFBP-3 or may be due to the low levels of transgene expression in these tissues at critical stages during the development.

1.5 Regulation of IGFBP-3

1.5.1 Physiological Conditions

Unlike IGFBP-1, IGFBP-3 is more stable and does not exhibit diurnal variation nor is it subject to postprandial change (Blum et al., 1993; Schwander et al., 1993). Calorie restriction decreased serum IGFBP-3 levels siginificanly in both children and adults, but protein restriction caused a decrease only in adults (Smith et al., 1995; Pucilowska, 1993). However, the decreased serum IGFBP-3 was normalized after protein refeeding. Weight loss or long-term moderate energy restriction does not affect IGFBP-3 (Rasmussen et al., 1994). Serum IGFBP-3 levels were not influenced by a very low caloric diet consumed by normal and obese subjects, while IGFBP-1 increased markedly in controls on very low caloric diet and not in obese subjects (Rasmussen et al., 1995). Taaffe and co-workers reported that serum IGFBP-3 levels were increased in adults after exercise (Taaffe et al., 1994). This increase was consistant with an increase in IGFBP-3 proteolytic activity. It is suggested that the proteolysis was induced by activation of

calcium-dependent protease (Lamson et al ., 1993).

1.5.2 Development and aging in humans

The IGFBP-1 and IGFBP-2 are the predominant binding proteins during the fetal life, and these two binding proteins decline during the early neonatal period with increasing of IGFBP-3. During fetal life, both the total amount of plasma IGFBP-3 and the amount of IGF-I and IGF-II bound to IGFBP-3 are lower than that in adults. IGFBP-3 level is low at birth, but it increases rapidly during the first years of infancy, and reaches a peak at puberty, declines during adulthood (Argente et al., 1993; Juul et al., 1995). IGFBP-3 level increases with pubertal maturation. Both the height and body mass index of an individual correlates positively with IGFBP-3 levels independent of age, sex and pubertal stage. The molar ratio between IGF-I and IGFBP-3 increases during puberty, indicating that more biologically active IGF is available in the free form during the pubertal growth spurt. Serum IGFBP-3 declines with age during adulthood, and this corresponds to an age-related decline in GH secretion (Blum et al., 1990; Baxter et al ., 1986).

1.5.3 Pregnancy

Serum IGFBP-3 is the predominant binding protein for cicurlating IGFs in both pregnant and nonpregnant women (Wang et al., 1992; Gargosky et al., 1991). Serum

IGFBP-3 levels were found lower by Western blot analysis in pregnant women (Guidice et al., 1990; Hossenlopp et al ., 1990). Using competitive binding studies, Binoux et al (Binoux M et al ., 1994) found that IGFBP-3 derived from material serum had 10 times lower affinity for IGF-I than IGFBP-3 derived from normal human serum and half the affinity for IGF-II, indicating the free form of IGFs would increase to meet physiological demand. The changes of serum IGFBP-3 in pregnancy is believed to be related to proteases produced in pregnancy (Davenport et al., 1990; Guidice et al., 1990; Hossenlopp et al., 1990).

1.5.4 Hormone and Growth factors

Serum levels of IGFBP-3 are regulated by IGF-I and GH under normal conditions (Blum et al., 1990). Administration of dexamethasone to rats resulted in a time- and dose- dependent increase in IGFBP-3 mRNA abundance and the 39-42kDa serum IGF binding proteins which most probably represent IGFBP-3 (Luo and Murphy, 1990). Previous studies showed that IGFs themself can increase levels of IGFBP-3 in vivo (Zapf et al., 1989; Jorgensen et al ., 1991). Direct addition of IGF-I stimulates the release of IGFBP-3 from a variety of cells in vitro (Conover, 1990; Conover, 1991; Smith et al., 1990). The study conducted by Betty et al (1996) demonstrated that insulin stimulates IGFBP-3 gene transcription but provides proportionally greater increases in IGFBP-3 release, whereas IGF-I alters IGFBP-3 expression by decreasing IGFBP-3 mRNA degradation.

1.5.5 Pathological conditions

1.5.5.1 Diabetes

IGFBP-3 reduction was reported in diabetic patients before insulin treatment (Batch et al., 1991; Bereket et al., 1995). After treatment, there was an increase in both serum IGF-I and IGFBP-3, with IGF-I showing a slight greater increase than IGFBP-3. IGFBP-specific protease(s) is increased in serum in some pathological states, which may lead to an increase in amount of free IGF-I available for growth stimulation. Ligand blot analysis of children with untreated IDDM showed that the intact IGFBP-3 was 50% lower than that of age-matched control while immunoassay detected 70% lower of IGFBP-3 (Gibson et al., 1995). After insulin treament, IGFBP-3 levels increased significantly, with an associated decline in serum IGFBP-3 protease (Bereket et al., 1995). Lower level of IGF-I is considered to be responsible for the decrease of IGFBP-3 in IDDM.

1.5.5.2 Tumors

IGFBP-2 is elevated in serum of patients with prostate cancer, whereas IGFBP-3 is decreased significantly (Cohen et al., 1993; Kanety et al., 1993). A decrease in IGF-I or the activation of a protease specific for IGFBP-3 was suggested for the decrease of IGFBP-3. It is possible that the decrease in IGFBP-3 may result in an increase in the

amount of free IGF-I in the target tissues, which promotes growth. Children with highly malignant CNS tumors or CNS leukemia have elevated IGFBP-3 in CSF possibly due to the disruption of the blood-barrier and entry of serum IGFBP-3 or to an increase in local production of IGFBP-3 by tumor tissues. Serum IGF-I and IGFBP-3 were decreased after chemotherapy for cranial tumors (Muller et al., 1993; Nivots et al., 1994).

1.5.5.3 Growth hormone Defect

Laron type dwarfism (LTD) is an autosomal recessive condition with a defect in the GH receptor gene. High level of growth hormone (GH) and low level of IGF-I are seen in the circulation of patients with LTD. Low level of IGFBP-3 was reported in LTD patients compared with normal controls and most of IGF-II in circulation appeared bound to IGFBP-2 instead of IGFBP-3 (Cotterill et al., 1992), and an IGFBP-3 specific protease was identified in serum of LTD patients. Lower level of IGFBP-3 in LTD patients may be due to the low level of IGF-I. Long-term treatment of LTD with IGF-I resulted in significantly progressive increase in serum IGFBP-3 levels (Kanety et al., 1993; Savage et al., 1993). This increase in serum IGFBP-3 indicates that IGFBP-3 in humans may be regulated directly by IGF-I, independent of GH.

In growth hormone deficiency (GHD) patients, GH level and serum IGFBP-3 level were low (Hall et al., 1988; Hardouin et al.,1989). Also in patient with hypopituitarism caused by certain tumors, low levels of IGFs and IGFBP-3 were found.

It is known that the serum levels of IGFBP-3 are dependent upon GH and / or IGF-I production. Regulation of IGFBP-3 by IGF-I and GH is mediated via an increased synthesis of IGFBP-3 or an alteration in IGFBP-3 breakdown (Lee, 1994; Hasegawa, 1995).

IGF-I can induce IGFBP-3 level independent of GH. This was confirmed by a study in which IGFBP level-3 in serum was increased in GHD patients after treatment of recombinant of IGF-I (Wilson et al., 1995).

Chapter 2. LITERATURE REVIEW OF GENE TARGETING

2.1 Gene targeting

Gene targeting is homologous recombination of DNA sequences residing in the chromosome with newly introduced DNA sequences (Mansour et al., 1988). Since the establishment of embryonic stem (ES) cells which are derived from the inner cell mass of mouse in 1981 (Evans et al.,1981; Martin ., 1981), gene targeting in the ES cell has gained tremendous development in the past decade. The general processes of gene targeting are as following: Recombinant DNA technology is used to alter a cloned DNA sequence of chosen locus, the modified DNA is then introduced into ES cells by transfection. The homologous recombination between the exogenous and endogenous chromosomal sequences occurs in ES cells and this mutation will integrate into the genome. ES cells carrying mutated locus are injected into mouse blastocyst from which germ-line chimeras can be generated. Interbreeding between heterozygous siblings will produce homozygous mouse for that desired mutation. Characterization of the homologous mice is carried out to elucidate the function of the interest gene. By using this technology, now it is possible that the designed manipulation of genes of interest can be achieved and that predetermined alterations in the mouse genome in order to study gene function and regulation can be obtained. A successful targeting experiment requires knowledge of the mouse gene structure, transcription start site (S), and potential for alternative splicing.

Gene targeting is composed of many steps, it can take a significant amount of time. Technically microinjection is the most difficult part in gene targeting. It requires specific skills and expensive equipment. The first gene targeting was accomplished in the β-globin gene in erythroleukemia by Smithies et al in 1985 (Smithies et al., 1985). Since then, gene targeting has been widely used, especially in ES cells, to make a variety of mutations in many different loci so that the phenotypic consequences of specific genetic modifications may be assessed in the organism. In order to better understand this technology, the following concepts and procedures are described in detail.

2.2 Targeting Vector

A targeting vector is designed to recombine and mutate a specific chromosomal locus. The basic components of such a vector are homologous sequences to the desired chromosomal integration site and a plasmid backbone (Joyner, 1993). The frequency of homologous recombination is improved when the genomic DNA uesd for targeting vector is isogenic to the target locus. Thus constructing the targeting vector from the same mouse strain the ES cell line is derived from is recommended. In order to increase transfection and the targeting frequency, usually positive and negative selection markers are needed in the targeting vector. The positive selection marker is usually placed between the flanking regions of homology. The positive selection marker has two functions. The first function is as a selection marker to isolate the rare transfected cells that carry the integrated DNA. The most commonly used positive selection marker is neomycin resistant

gene (Neo^r). The second function is as a mutagen to replace the exons of the gene. The negative selection marker serves as selection for random integration (Mansour et al., 1988), the commonly used negative selection marker is herpes simplex virus thymidine kinase (HSV-TK) gene which is usually inserted at the end of the targeting vector (Mansour et al., 1988).

2.3 Introduction of DNA into ES cells and ES cells culture

DNA (targeting vector) is usually introduced into ES cells by electroporation which delivers high voltage electrical pulse to a suspension of cells and DNA (Potter et al., 1984). After application of this pulse, the DNA passes through pores in the cell membrane into the ES cells. About 50% of cells die of this procedure which is the optimal transfection efficiency. The factors influencing the efficiency and cell viability are the voltage, the ion concentration, the DNA concentration and the cell concentration (Joyner , 1993). ES cells are derived from inner cell mass of a mouse blastocyst. They are passaged, frozen and thawed as normal cell lines. However, the distinct property of ES cells is that they can be manipulated genetically in vitro and retain the potential to participate in normal embryonic development and are able to differentiate into all of the cell lineages including germ-line cell, the genotype of the ES cell that was genetically manipulated will pass on to the next generation essentially creating a "new" strain of mouse. ES cells are usually maintained in an undifferentiated state on a feeder layer of an embryonic fibroblast cell line or in the culture medium supplemented with leukemia

inhibitory factor (LIF) (Evans et al., 1981; Martin et al., 1981) which can inhibit the differentiation of ES cells. However, culture conditions and passage number may influence the ability of ES cells to give to germ-line chimeras.

2.4 Homologous recombination and selection of targeted events

Gene targeting is achieved through a process of homologous recombination between a mutant construct and an endogenous gene. Homologous recombination is a rare event in eukaryotic cells. Johnson et al (1989) reported homologous recombination frequencies between 1 in $5x10^7$ and 1 in 6.6 x 10^6 cells transfected with targeting constructs. With so low targeted frequency, the major technical difficulty in gene targeting has been the unavailability of practical methods for obtaining ES cell lines carrying desired targeted mutations at loci of interest. Mansour et al (1988) designed a positive and negative selection procedure which was reported to enrich 2,000-fold for those cells that contain a targeted mutation. It has been the most commonly used method for screening ES cells with desired mutation. In this method, Neo^r is used as a positive selection marker, and is inserted between the flanking regions of homology. As described above, Neo^r serves two functions here. First, to disrupt the coding exons of the interest gene, second as a selective marker for ES cells (conferring resistant to the drug G418) containing the integrated copy of the recombinant vector. The negative selection marker, HSV-TK, is usually placed at the end of the construct outside the region of homology. The targeting vector is so constructed that when replacement of the endogenous sequence

by the exogenous one occurs via homologous recombination, the HSV-TK gene will not be transferred into the endogenous target. Exclusion of the HSV-TK gene during the homologous recombination occurs, because the HSV-TK gene represents a discontinuity in the incoming vector between homology and non-homology with the endogenous target sequence. Cells in which the targeting events occurred will therefore be Neo^r and HSV-tk^-. On the other hand, random integration of the targeting vector into the recipient cell genome should result in most cases, in cells that are Neo^r and HSV-TK^-. For the positive selection, the most commonly used drug is G418, which will kill ES cells without expression of neo^r gene. For the negative selection, the drug gancyclovir is usually used. Gancyclovir is a kind of toxic thymidine analogs that is utilized by the viral enzyme much more efficiently than by the cellular enzyme (Sedivy and Joyner, 1992), therefore ES cells containing HSV-TK (HSV-TK^+) will be killed by gancyclovir in the medium. The combination of positive and negative selections will select homologous recombination as ES cells are both resistant to G418 and gancyclovir. The positive and negative selection makes it feasible to enrich for homologous recombination events and subsequently detect cellular clones in which the gene has been disrupted as reported (Mansour et al., 1988).

2.5 Research Objectives

Many studies on the function of IGFBP-3 have been conducted in the last decade, the results are inconclusive. The physiological function of IGFBP-3 has not been precisely

defined. So far only one transgenic model which expressed human IGFBP-3 was developed (Murphy et al., 1995), and the phenotype in this model was normal. The development the gene targeting technology in ES cells provides a powerful way to study the function of specific genes in the mouse. In an attempt to study the physiological function of IGFBP-3 and explore its undiscovered functions, homologous recombination gene targeting technology was utilized to generate a IGFBP-3 null mutant mice model.

Chapter 3. MATERIALS AND METHODS

3.1 Materials

Restriction enzymes and DNA modifying enzymes were purchased from Pharmacia (Canada) Inc.(Baie d'urfe, Quebec), Gibco/BRL Life Technologies Inc. (Burlington, Ontario), Boehring-Mannheim (Laval, Quebec), New England Biolabs Inc. (Beverly, Massachusetts) and Promega Biotec. (Madison, Wisconsin). [Alpha-^{32}p] dCTP was supplied by ICN Biochemical Canada Ltd. (Oakville, Ontario) and Amersham Canada Ltd. (Oakille, Ontario). Nick translation Kits were purchased from Amersham Canada Ltd. G418 was purchased from GIBCOBRL. Grand Island, New York. Gancyclovir was obtained from Syntex Inc. Mississanga, Ontario. Mouse embryonic stem cells (R1), mouse genomic library (Lambda Dash 129 library) and Herpes simplex virus thymidine cassette (plasmid pPNT) were kindly provided by Dr. J Rossant, Samuel Lunenfeld Institute, University of Toronto, Toronto, Ontario. Leukemia Inhibitory factor (LIF) and Dubecco' Modified Eagle Medium were supplied by Life Technologies, Grand Island, New York. Mice (CD1) were supplied by the Central Animal Care, Faculty of Medicine, University of Manitoba, Winnipeg.

3.2 Labelling of probes

3.2.1 Nick-translation

Gel-purified DNA fragments were labelled with [^{32}p] by nick translation reaction (Feinberg et al., 1983). In this labelling method, the DNA polymerase I initiates DNA synthesis at free 3' OH groups. The nicks are produced at random locations by digestion of DNaseI. The nick translation reaction was carried out as described by the supplier of the kit. Usually 7ul of 3,000Ci/mmol [alpha-^{32}p] dCTP and 100-200ng of purified DNA was used per reaction. Radioactively labelled DNA was separated from unincorporated [alpha-^{32}p] dCTP by G100 sephadex gel filtration chromatography.

3.2.2 End labelling

5' ending labelling was used for the synthetic oligonucleotides. The T4 polynucleotide kinase can specifically transfer the gamma ^{32}p label from ATP to a 5' OH group of DNA. Usually 300ng oligonucleotide was incubated at 37^{0}C for 30 minutes with T4 polynucleotide kinase and [gamma-32p] in the presence of a kinase buffer (500 mM Tris, pH 7.4, 100mM $MgCl_2$, 50 mM dithiotreitol (DTT), 10 mM spermidine. The reaction was stopped with 0.5 M EDTA, pH 8.0 (28mM final). The unincorporated label was removed by DE-52 column and labelled oligonucleotide was eluted by high salt solution (10 mM Tris, PH 7.5, 500 mM NaCl, 0.2 mM EDTA).

3.3 Large scale preparation of plasmid DNA

Plasmid amplification was prepared by using the method described by Maniatis et

al 1984 with some modifications. A single colony carrying the plasmid was incubated in 10ml of LB medium containing ampicillin. After overnight culture at 37°C shaker, the bacterial medium was transferred into 2 liter flask containing 500ml of LB medium with ampicillin and continued incubating until the bacterial cells reached 0.4-0.6 (O.D 600). 2.5 ml of chloramphenicol (34 mg/ml in ethanol) was then added to the culture and continued shaking at 37°C for a further 16 hrs. The medium was then centrifuged at 3000 rpm (4°C) for 10 min in PR 7000 (International Equipment Company, Needham Hts, MA). The supernant was discarded and the bacterial pellet was resuspended in 10 ml of lysis buffer (25mM Tris, PH 8.0, 10 mM EDTA, 0.9% glucose, 5mg/ml lysozyme) on ice for 15 min. 10 ml of 0.2N NaOH, 0.2% SDS solution was added and placed on ice for 15 min. Then 10 ml of 3M sodium acetate PH5.2 was added and the mixture was left on ice for 30 min. The mixture was centrifuged in a JA-20 rotor at 15,000 rpm for 40 min at 4°C. The supernant was transferred into a 50 ml corning tube and 0.6 volume isopropanol was added for precipitation of DNA. The DNA was collected by centrifugation at 3,000 rpm in PR 7000 for 20 min at room temperature. The pellet was then dissolved in 6.0 ml of TE pH 75, finally 6.6 Cesium Chloride and 0.5 ml of ethidium bromide (10 mg/ml) was added to the mixture. After well-mixed, the mixture was ultracentrifuged at 55,000 rpm in a Beckman Ti75 rotor at 20°C for 18 hrs. After ultracentrifugation, the lower plasmid band was collected under long ultraviolet light. The collected material was extracted with isoamyl alcohol 3 times or more to remove the ethidium bromide. Two ml of sterile water was added and followed by 2.5 volumes of cold ethanol and the mixture was kept at -20°C overnight. The mxiture was then

centrifuged at 10,000rpm at 4°C for 40 min the pellet was washed with 70% of ethanol and dissolved in TE or distilled water.

3.4 Subcloning

The desired DNA fragment was digested with appropriate restriction enzymes and separated by gel electrophoresis, isolated by electroelution as described by Maniatis et al 1984. The vector was digested to produce compatible ends with the fragment to be subcloned. In the ligation reaction, the concentration of insert DNA was relatively high in order to facilitate the ligation to the vectors. The molar ratio of inserted vector was 3:1. In order to reduce background, vector DNA cleaved with enzymes was treated with calf intestinal alkaline phosphatase (CIAP). Although CIAP treatment lowers the absolute efficiency of cloning the desired DNA molecule, the great reduction in background greatly enhances the isolation of the desired clone (Dugaiczyk et al., 1975; Struhl et al., 1985). 1-6 units of T4DNA ligase was used in a total volume of 20ul. The reaction was allowed to proceed overnight at 15°C. 5-20ul of reaction was introduced into competent cells (E.coli.JM109). The transformed cells were plated on agar plates which contained 100ug/ml ampicillin. The recombinant clones were selected by ampicillin resistance.

3.5 Mini-preparation of plasmid

The alkaline lysis procedure was used for the isolation of small quantities of

plasmid DNA from bacterial cells (minipreps) described by Birnboim and Doly 1979 with modification. The ampicillin resistant colonies were picked up into 1.5ml LB medium with ampicillin in glass tubes with silver cover. The bacteria were incubated in a shaking incubator overnight at 37°C. The culture was centrifuged at 9,000rpm at 4°C. The pellets were suspended in 100ul lysis buffer (25 mMTris-HCI PH 8.0, 10 mM EDTA-2Na, 50 mMD-glucose) for 5min. Then a 200ul fresh prepared alkaline solution (1% SDS, 0.2 N NAOH) was added. The tubes were mixed by inverting rapidly three times and stored on ice for 5min. 150ul of 3M sodium acetate PH 5.6 was added. The mixture was centrifuged at 10,000 rpm for 10min. The supernant was extracted with phenol/chloroform and precipitated with cold ethanol. The DNA pellet was dried under vacuum and dissolved in TE or water for analysizing.

3.6 Genomic library screening

Phage growth and purification were performed using the method described by Maniatis T (1984) and genomic library screening was performed using the protocol supplied by Stratagene (1993) with some modifications. First, the genomic library was tested to determine an optimal concentration for screening by growthing in a series of dilutions with the host bacteria in the LB supplemented with 20% maltose and 1M of Mg_2SO_4. Phage was diluted in a SM buffer [1 litre of SM contains 5.8g of NaCI, 2.0g of $MgSO_4.H_20$, 50.0 ml of 1 M Tris-HCI pH 7.5, 5.o ml of 2% (W/V) Gelatin]. The phage at the optimal concentration was incubated with host bacteria at 37°C for 20 min

in the glass tubes with silver caps. The mixture was then mixed with 3.5ml of top agar (48^0C) and plated on 100mm plates and the plates were incubated up to 12hr. After incubation, the plates were kept at 4^0C for at least 2 hrs to allow the plates to dry. The plates were transferred onto the nitrocellulose filters for 1 min to allow phage to transfer onto the filters and the asymmetrical marks were made on the plates with needle for orientation. After transfer, the nitrocellulose filters were denatured for 1-2 min in the denature solution and neutralized in the neutralizing solution for 1-2 min as described in Southern blot hybridization.The filters were then washed for 1-2 min in a 6x SSC solution and dried. The prehybridization and hybridization were performed as same as in Southern blot hybridization. After autoradiography, the films were oriented to the filters according to the marks. The putative clones were determined by the strongest signal on the film. A square centermeter piece of agar from the stock plates where the putative clones lined up with film spots was cut out, and the plaque was isolated by pasteur pipette and dissolved in 0.5ml of SM buffer. Dilution of phage, prehybridization and hybridization were performed as before. This is the second screening. For the tertiary screening, a isolated plaque from the second screening was picked up. The isolated plaque was incubated with host bacteria on the plate for up to 12 hrs. When all plaques in a plate were positive, then amplification and purification of phage DNA were carried out using the methods as described by Manistis T (1984).

3.7 ES cell culture

ES (R1) cell were cultured in Dulbecco's Modified Eagles Medium (DMEM) with 4.5g/L glucose, 10^{-4} M 2-mercaptoethanol, 50ug/ml of penicillin and streptomycin and supplemented with 15% heat inactivated Fetal Calf Serum (FCS) in a constant environment of 37°C, 5% CO_2 and 100% humidity (Thomas et al., 1987; Joyner, 1993). ES cells were maintained in leukemia inhibitory factor (LIF)during most of time of this study. At later stage of the study, both feeder layer of primary embryonic fibroblast (EMFI) cells and LIF were used in the culture of ES cell (Joyner, 1993). LIF has been used successfully in the culture of ES cells instead of feeder layer (Evans et al., 1981; Martin et al., 1981). Both feeder layer and LIF function as inhibitiors of differentiation of ES cells, because ES cells must be maintained in undifferentiated state so that they can contribute to development of the embryos. 10^{-3} units/ml of LIF was used in the culture of ES cells as recommended by the supplier. ES cells were examined daily and passaged exactly every 2 days. Subcultivation ratio was 1:5 to 1:10.

3.8 Transfection and selection of ES cells

DNA of the targeting vector was introduced into ES cells by electroporation using method described by Joyner (1993), which was conducted in gene pulser electroporator (Bio-Rad). The set up for the electroporation was 240V and 500uf. ES cells were harvested by trypsinization. The density of the ES cells was adjusted to 7 x 10^6/ml and suspended in a PBS buffer for ES cell (1 litre of this buffer contains 10g NaCl, 0.25g KCl, 1.44g Na_2HPO_4 and 0.25g KH_2PO_4.pH7.2). The IGFBP-3 targeting vector linearized

by Not1 was added to the ES cells at final concentration of 40-50ug/ml prior to electroporation. Aliquots (0.4 ml) of ES cells containing DNA of targeting vector were electroporated in cuverttes (0.4ml each) in the gene pulser. After electroporation, the ES cells were placed on ice for 20min, and then equally distributed into three 10 cm culture dishes.

The positive and negative selection method (Mansour et al., 1988) was applied to screen homologous recombination events from a subset of transfected ES cells with targeting vector. The neomycin resistant gene (Neo^r) which is positive marker confers resistance to the antibiotic G418. The ES cells that contain Neo^r will survive the positive selection. The negative selection marker is the herpes simplex virus thymidine kinase (HSV-TK) gene which kills the ES cells that express HSV-TK in the presence of gangcyclovir.

To allow the expression of Neo^r gene and HSV-TK gene, the ES cells were first cultured on three 10 cm culture plates in nonselective medium after electroporation and refed the next day. G418 and gancyclovir were added to the culture medium at concentrations of 200μg/ml and 2μM respectively 48 hrs after electroporation. The ES cells were refed every 3 or 4 days and the resistant colonies gradually appeared. After 7-9 days culture in selection medium, the resistant colonies were picked up.

Each colony was treated with 30μl of trypsin (0.25% trypsin and 0.57mM EDTA

) and transferred into one well of 96 well plates for a further growth of 5-7 days. By this time most ES cell cultures were confluent and transferred into 24 well plates for growth. After 3-4 days of growth, the cell cultures became confluent. Then the ES cell culture were split into two sets. One was used for extraction of DNA which was subjected to screening for homologous recombination. Another one was continue to culture until confluent and frozen and storaged at -70^{0}C or in liquid nitrogen for long term (Joyner, 1993), this set of ES cells was kept for blastocyst microinjection once the homologous recombination was confirmed.

3.9 Preparation of ES cell DNA

The ES cells were washed twice with PBS in the 24 well of plate, then 500μl of lysis buffer [100mM NaCl, 100mM Tris.HCl, pH8.0, 25 mM EDTA pH 8.0, 0.5% sodium dudecyl sulfate (SDS), 0.2 mg/ml proteinase K] was added to each well and aspirated several times until the ES cells were lifted. The lysate of ES cells was transferred to a 1.5ml microcentrifuge tube and incubated overnight at 55^{0}C. After incubation, the lysate was extracted with phenol and phenol/chloroform, the DNA was precipitated with cold 95% ethanol, the pellet was washed with 70% ethanol and dried with vacuum. Finally the DNA pellet was dissolved in TE (pH 8.0) or sterilized distilled water for further analysis.

3.10. Southern blot hybridization

DNA samples were digested with appropriate restriction enzymes, and then separated by gel electrophoresis in a 1% agarose gel along with DNA size markers. A photograph of gel was taken for the record. The gel was first denatured in a solution of 1.5 M NaCI and 0.5 M NaOH for 20 min and then neutralized in a solution of 0.5 M Tris and 3.0 M NaCI pH 7.0 for 40min. The DNA gel was then transferred to nitrocellulose filters (Nitro plus) in 20 x SSC (3M NaCI, 0.3M sodium citrate) overnight. The blot was dried at 80^0C for 2 hrs or 60^0C overnight. Nitrocellulose filter with transferred DNA was prehybridizied for at least 2 hrs at 42^0C in a hybridization solution (50%formamide, 20mM NaH_2PO_4 pH 7.0, 4x SSC (1x =0.15M NaCI, 0.015 sodium citrate), 2mM EDTA, 4x Denhardt' solution (1x=0.02%BSA, ficoll, and polyvinylpyrrolidine), 0.1% SDS and 100μg/ml sonicated, denatured salmon sperm DNA. Hybridization was performed at 42^0C in the hybridization solution with the DNA probe. After hybridization, the blots were washed first at room temperature with 2 x SSC and 0.1% SDS for 30 min and further washed with 0.1%SDS and 0.2 x SSC at 65^0C for 15min or longer depending upon radioactivity in the filters. Autoradiography was performed by exposing Kodak X-Omat AR film to nitrocellulose filters in the presence of an enhancing screen at -70^0C for 1hr to several days depending on radioactivity of the film. Finally the films were developed for analysis.

3.11. Preparation of animals and collection of blastocyst

Superovulation was used in the production of embryos (Joyner, 1993). Briefly, 2-10 units of pregnant mare serum gonadotropin (PMSG, Sigma) were injected intraperitoneally to CD1 female mice between noon and 4 pm on day one followed by an injection of 2 - 10 units of human chorionic gonadotropin (HCG, Sigma) at noon of day three. Then the female were caged with stud males. They were checked for the plugs in the vaginas the next morning. The presence of vaginal plug is the indication of successful mating. The pseudopregnant females (foster mother) that serve as the recipient of embryos after microinjection were obtained by mating with vasectomized males one day later than the females mice treated with the hormone. Pregnant females were killed in the morning on the fourth day of pernancy. Blastocysts were flushed out from the uteri of pregnant females by using needles filled with M2 meduim as described by Joyner (1993). After three washs with M2, the blastocysts were cultured in M16 medium at 37^0C for microinjection. (Joyner et al., 1993)

3.12 Blastocyst microinjection

ES cell in the exponential growth phase were fed and trypsinized one hour later, maintained as a single cell suspension in M2 or PBS on ice. The set up of micromanipulator was followed the the procedure described by Joyner (1993). The holding and injecting pipettes were prepared in advance. The injection pipette was filled

with mineral oil. During the microinjection, the first step was to pick up some small round ES cells with the injection pipette. Then blastocysts were held with holding pipette by suction and were positioned so that the whole inner cell mass was on the side of holding pipette and blastocoelic cavity was facing to the injection pipette. The injection pipette was positioned by the joystick and penetrated through trophectoderm by a sudden, fast force imposed by joystick. 10-20 ES cells were gentlely expelled into each blastocoelic cavity. After microinjection, the blastocysts were moved back to incubator for 1.5-2 hrs incubation before being transferred into uteri of foster mothers.

Chapter 4. RESULTS

The human IGFBP-3 gene has already been cloned (Wood et al., 1988; Cubbage et al., 1990). The mouse IGFBP-3 gene has not yet been cloned. The cDNA of mouse IGFBP-3 has been reported (Schuller et al., 1994); the homology of amino acid sequences between mouse and human IGFBP-3 was 81%. Therefore in my study, different fragments prepared from human IGFBP-3 cDNA (provided by Dr Wood, Department of Development Biology and Molecular Biology, Genetech Inc. South San Francisco, California) were used to characterize the mouse IGFBP-3 gene. The human IGFBP-3 gene and human IGFBP-3 cDNA maps are shown in Figure 1.

4.1 Isolation and Purification of mouse IGFBP-3 gene

A Lambda Dash 129 Library (provided by Dr Rossant, Samuel Lunenfeld Research institute, University of Toronto, Toronto, Ontario) were screened by using a full length of human IGFBP-3 cDNA labelled with ^{32}P as a probe. A total of about 2×10^7 plaques were screened. One positive plaque was identified to have mouse IGFBP-3 gene by hybridizing with a full length of human IGFBP-3 cDNA. Second screen was amplified from the first one and extended to the tertiary screen. When all the plaques in a plate were positive ones, then an isolated plaque was picked up and amplified for extraction of phage DNA. The dark spots in Figure 2 are the positive plaques. The positive phage plaques were amplified by growing with host bacteria. The DNA was

extracted from amplified phage according to the method as described by Maniatis et al (1984). Phage DNA purified from the positive plaques was digested with different enzymes and subjected to Southern blot hybridization analysis (Figure 3).

4.2 Analysis of mouse IGFBP-3 gene

A 8.0 kb BamHI fragment was seen on the agarose gel after enzymatic digestion of phage DNA containing the mouse IGFBP-3 gene. It showed a very strong signal when hybridizing with human IGFBP-3 cDNA as indicated in Figure 3 (B). Only one BamHI fragment was found to hybridize with human IGFBP-3 cDNA on the BamHI digestion lane of the mouse IGFBP-3 phage. Thus the BamHI fragment was selected for the construction of targeting vector. In Figure 3, a KpnI and a NotI fragments were seen both on the agarose gel and on Southern blot hybridization. It indicated that only one Kpn **I** restriction site and one NotI restriction site are present in the mouse IGFBP-3 gene.

The 8.0kb BamHI fragment was subcloned into p Bluescript SK (Stratagene) as showed in Figure 4(A). A 8.0 kb of BamHI fragment and 3.0 kb of vector were seen on the gel as indicated by the arrows. In Figure 4 (B), Southern blot analysis confirmed the 8.0 fragment was the BamHI fragment by hybridizing with a probe containing part of exon 1 and exon2-4 of human IGFBP-3 (refer to the map of human IGFBP-3 in Figure 1). Only two bands were found in the KpnI digest lane indicating only one KpnI restriction site was present in this fragment as shown in Figure (C), the small band was

the KpnI fragment and the big band was the vector plus BamHI-KpnI fragment (refer to Figure 6). Southern blot hybridization showed that only the 3.8 kb of the KpnI fragment hybridized with the probe containing exon1-4 of human IGFBP-3, but not the BamHI-Kpn I fragment. Therefore the BamHI-KpnI fragment could contain either the 5' of the exon 1 or exon 5 which is an uncoding exon in human IGFBP-3. In Figure 7A, the BamHI-KpnI fragment hybridized with a probe containing exon-5 of human IGFBP-3, suggesting it contains the exon 5 of the mouse IGFBP-3. That the BamHI-KpnI fragment did not hybridize with the probe containing exon1-4 of human IGFBP-3 also proved this point. The BamHI-KpnI fragment was selected as the 3' segment of the targeting vector for mouse IGFBP-3.

4.3 Identification of 5 ' and 3' fragments of mouse IGFBP-3

A BamHI/NotI fragment in the phage DNA containing mouse IGFBP-3 gene was identified as a 5' fragment of mouse IGFBP-3 (Figure 5 A) by hybridizing with SacII fragment (refer to Figure1) which only contains exon 1 of human IGFBP-3 and subcloned into p Bluescript SK (Stratagene) (Figure 5B). The BamHI/KpnI fragment did not hybridize with the SacI/SacII fragment which contains a part of exon1 and exon2-4 (Figure 4C). This together with Southern blot analysis in Figure 7A demonstrates that the BamHI/KpnI fragment only contains exon 5 of mouse IGFBP-3. The BamHI/KpnI fragment was chosed as the 3' fragment of targeting vector for the mouse IGFBP-3 and subcloned into p bluescript SK. A 6.2 kb of XhoI/KpnI fragment hybridizing with ScaII

fragment of human IGFBP-3 was isolated (Figure7 B) and subcloned into p Bluescript SK. Digestion of the BamHI/NotI fragment (5' fragment) and BamHI/KpnI (3' fragment) and XhoI/KpnI fragment together with a number of enzymes showed that there is a common fragment between the BamHI/NotI and the XhoI/KpnI fragments and a common fragment between the XhoI/KpnI and the BamHI fragments, and there is no common fragment between the XhoI/KpnI and the BamHI/KpnI fragments (Figure 8). Therefore an overlap between the BamHI/NotI and the XhoI/KpnI fragments and an overlap between XhoI/KpnI and KpnI exist. A schematic restriction map of mouse IGFBP-3 was established based on the results of restriction analysis above (Figure 9).

4.4 Construction of mouseIGFBP-3 targeting vector

The plasmid, pPNT was specifically designed for gene targeting (Figure 10) (provided by Dr Rossant, Samuel Lunenfeld Research Institute, University of Toronto, Toronto, Ontario). It has a Neo and a tk cassette in the plasmid for positive and negative selection and the expression of these two genes is driven by the mouse phosphoglycerate kinase-1 (PGK-1). For the purpose of subcloning, the 5' fragment BamHI/NotI in p Bluescript was modified to BamHI/SalI fragment by digesting with BamHI and SalI; and the 3'fragment BamHI/KpnI in p Bluescript was modified to EcoRI end by digesting with BamHI and EcoRI (refer to the map of p Bluescript, Stratagene). Both the 3' and 5' fragments were subcloned into pPNT subsequently (Figure 11). The transcriptional orientation of the mouse IGFBP-3 is the same as pPNT. Digestion of targeting vector

together with 5' and 3' fragments showed that the targeting vector released the same pattern bands as both the 5' and 3' fragments (Figure 12) demonstrating a mouse IGFBP-3 gene targeting vector was successfully constructed.

4.5 Gene targeting and analysis of ES cells

The strategy of mouse IGFBP-3 gene targeting was depicted in Figure 13. The targeting vector is composed of a 5' fragment (SalI/NotI fragment) and a 3' fragment (EcoRI/KpnI fragment) which contain exon 1 and exon 5 respectively. Neo^r is inserted between the two homologous sequences, whereas HSV-tk is placed outside of the homologous region. By homologous recombination, the sequences between exon 1 and exon 5 will be deleted and replaced by Neo^r gene. The mouse IGFBP-3 targeting vector was linealized at a unique NotI site and was introduced into ES cells by electroporation. The positive-negative selection was utilized for screening homologous recombination events in the ES cells. This selection enriches for homologous recombination events by simultaneously selecting for a Neo gene within the homologous DNA against a herpes simplex virus thymidine kinase (HSV-tk) gene placed at the end of the targeting vector. G418 and gancyclovir were used for the positive and negative selection for the ES cells after electroporation. The DNA extracted from control ES cells and from two targeted ES clones (clone 41 and 278) was digested with BamHI and subjected to Southern blot hybridization according to the strategy in Figure 13. In Figure 13 (A), the probe A was used to detect the mutant allele for mouse IGFBP-3, the 8.0 kb fragment is derived from

the wild-type allele whereas the 4.0 kb is the targeted allele. In 13 (B), a 6.3 kb fragment of the normal allele and a 3.3 kb of the mutant allele were detected by probe B. An average of 422 colonies were obtained from $1.5x10^7$ ES cells survived electroporation. Of 550 ES cell colonies screened by Southren blot hybridization, two was identified to have the homologous recombination for mouse IGFBP-3 locus, the average targeting frequency was 0.36% (Table 2).

TABLE 2 Results of screening for homologous recombination

Expt No.	No. of ES cell surviving electroporation	No. of G418 and GANC colonies	No. of colonies screened	No. of recombinant	Frequency (%)	Method of analysis
1	2×10^7	550	170	0	-	Southern
2	1.8×10^7	450	130	0	-	Southern
3	1.5×10^7	340	108	1	0.9	Southern
4	1.0×10^7	350	142	1	0.7	Southern

4.6 Generation of chimeric mice for IGFBP-3

The two ES cell lines 41 and 278 carrying IGFBP-3 mutant gene were microinjected into blastocysts of CD1 mice. Only one cell line (41) gave rise to chimeric

mice. A total of 1800 CD1 blastocysts were injected with targeted ES cells (clone 41). 96 mice were transferred with the injected blastocysts and 290 mice were born from the foster mothers. Out of 290 mice seven are chimeric mice which is determined by the coat color (Table 3). None of the chimeras was found to carry the germline transmission (Table 4). More chimeric mice will have to be generated to obtain the germline transmission in order to achieve null mutant mice of IGFBP-3.

TABLE 3 Chimera production

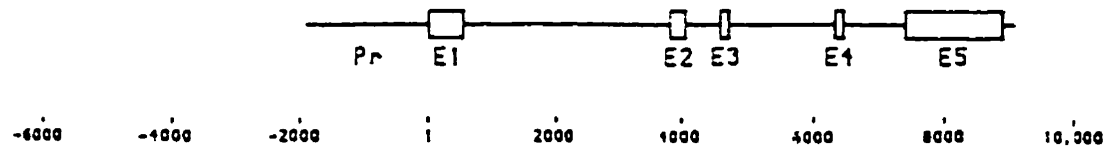
Targeted ES cell clone	Blastocyst strain	ES cell line	No. blastocyst injected	No. mice transferred	No. progeny	No. chimeraes
41	CD1	R1	1800	96	290	7
278	CD1	R1	600	30	85	0

TABLE 4 Chimeric mice generated

Mouse	Sex	% chimeric by coat color	No. of breeding	No. of heterozygous
1	F	30%	2	0
2	F	30%	2	0
3	M	50%	2	0
4	F	40%	2	0
5	F	40%	2	0
6	M	30%	2	0
7	M	30%	2	0

Figure 1. (A). Restriction map of human IGFBP-3 gene (Cubbage et al., 1990). The open boxes are exon 1-5 (E1-E5). The 5'-flanking region and promoter are presented as line labeled Pr to the left of exon 1, the four introns are represented as the lines between exons. The numbers at the bottom represented the distance in nucleotides, 5' (negative) or 3' (positive) to the mRNA cap site (nucleotide 1). (B) Restriction map of human IGFBP-3 cDNA (Wood et al., 1988). The open boxes are the encoding region.The closed box is the untranslated sequences. The restriction sites are labeled as indicated. The following fragments were prepared from the human IGFBP-3 cDNA for characterizing the mouse IGFBP-3 gene. SacII fragment: containing exon 1. position: +166- +439 . SacII/SacI fragment: containing 3'-sequence of exon1, exon 2-3, most sequences of exon 4. position: +439 - +1008 . PstI fragment containing exon-5. position: +1813-+2294.

(A)


Pr
E1
E2
E3
E4
E5
-6000
-4000
-2000
1
2000
4000
6000
10,000

(B)

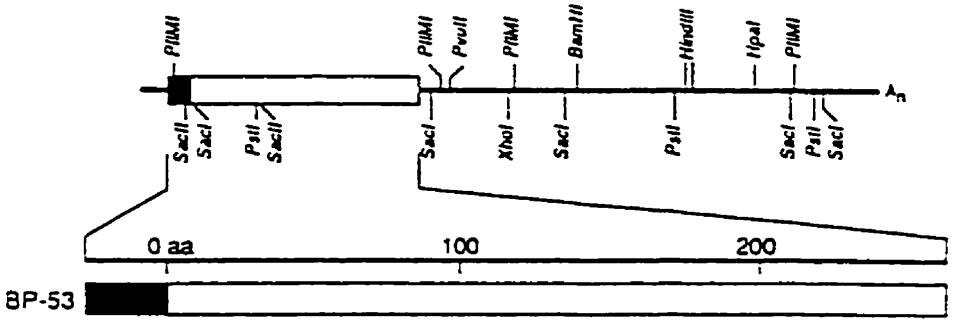
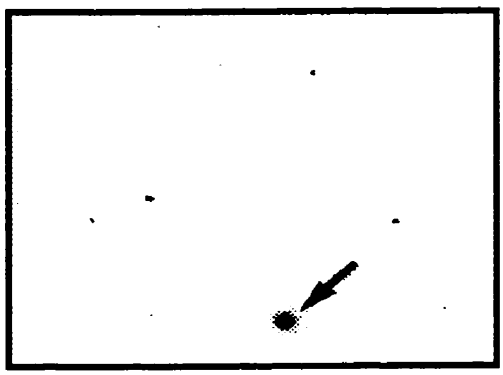
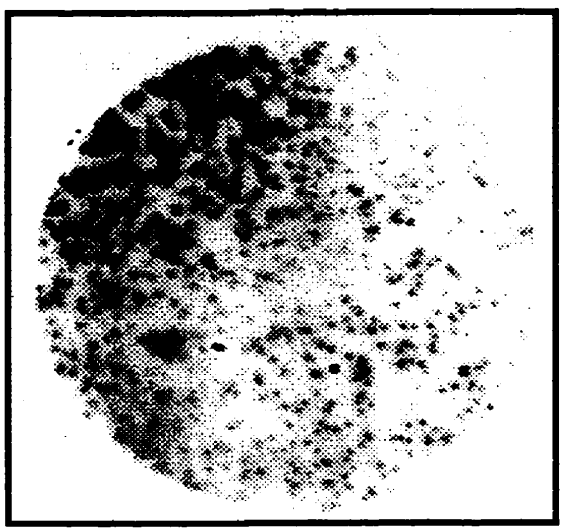

PflMI
PflMI
PvuII
PflMI
BamHI
HindIII
HpaI
PflMI
SacII
SacI
PstI
SacII
SacI
XhoI
SacI
PstI
SacI
PstI
SacI
An
0 aa
100
200
BP-53

Figure 2. Screening of mouse genomic library. The genomic library (Lambda Dash 129) in phage diluted in a concentration determined by a series of dilution were incubated with host bacteria in a LB medium supplemented with 20% maltose and 1M of Mg_2SO_4 for 20 min and grew on the agar plates for 16 hrs. The phage DNA was transferred to a round nitrocellulose membrane. The membrane was hybridized with a ^{32}P labeled full length of human IGFBP-3 cDNA. Hybridization was performed at 42^0C and the filters were washed in a final concentration of 2xSSC/1%SDS for 30 min at room temperature and in a final concentration of 0.2x SSC/1%SDS for 15min at 60^0C. Autoradiography was carried out for 24-48 hrs. The dark spots are positive plaques. The second screen was amplified from the first screen and third screen was amplified from the second one.

First Screening



Second Screening



Third Screening

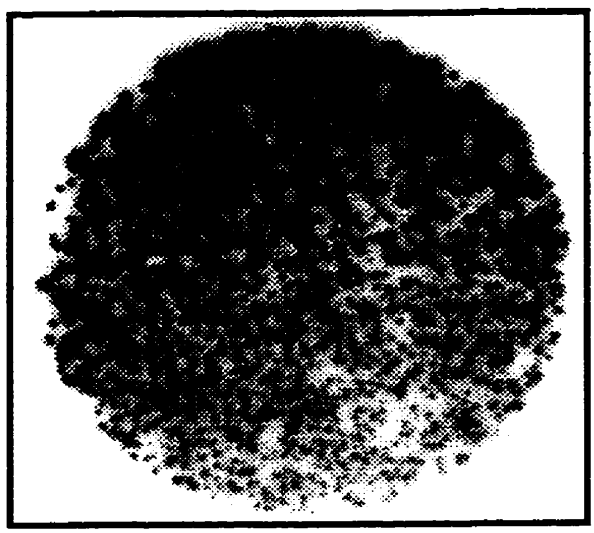


Figure 3. Digestion of phage DNA containing the mouse IGFBP-3 gene. Phage DNA from the positive plaques was digested with different enzymes as shown and separated in a 1.0% agarose gel, transferred onto a nitrocellulose membrane and hybridized with a ^{32}P labeled human IGFBP-3 cDNA. The hybridizing fragments were visualized by autoradiography. Each lane contains 30μg DNA. (A) Ethidium bromide stained gel of DNA. (B) Southern blot hybridization analysis . A single BamHI fragment is seen on the Southern blot hybridization as indicated by the arrow. This fragment was isolated and subcloned into p Bluescript SK for further analysis. The positions of the DNA size markers, HindIII-digested bacteriophage λ DNA, are also shown.

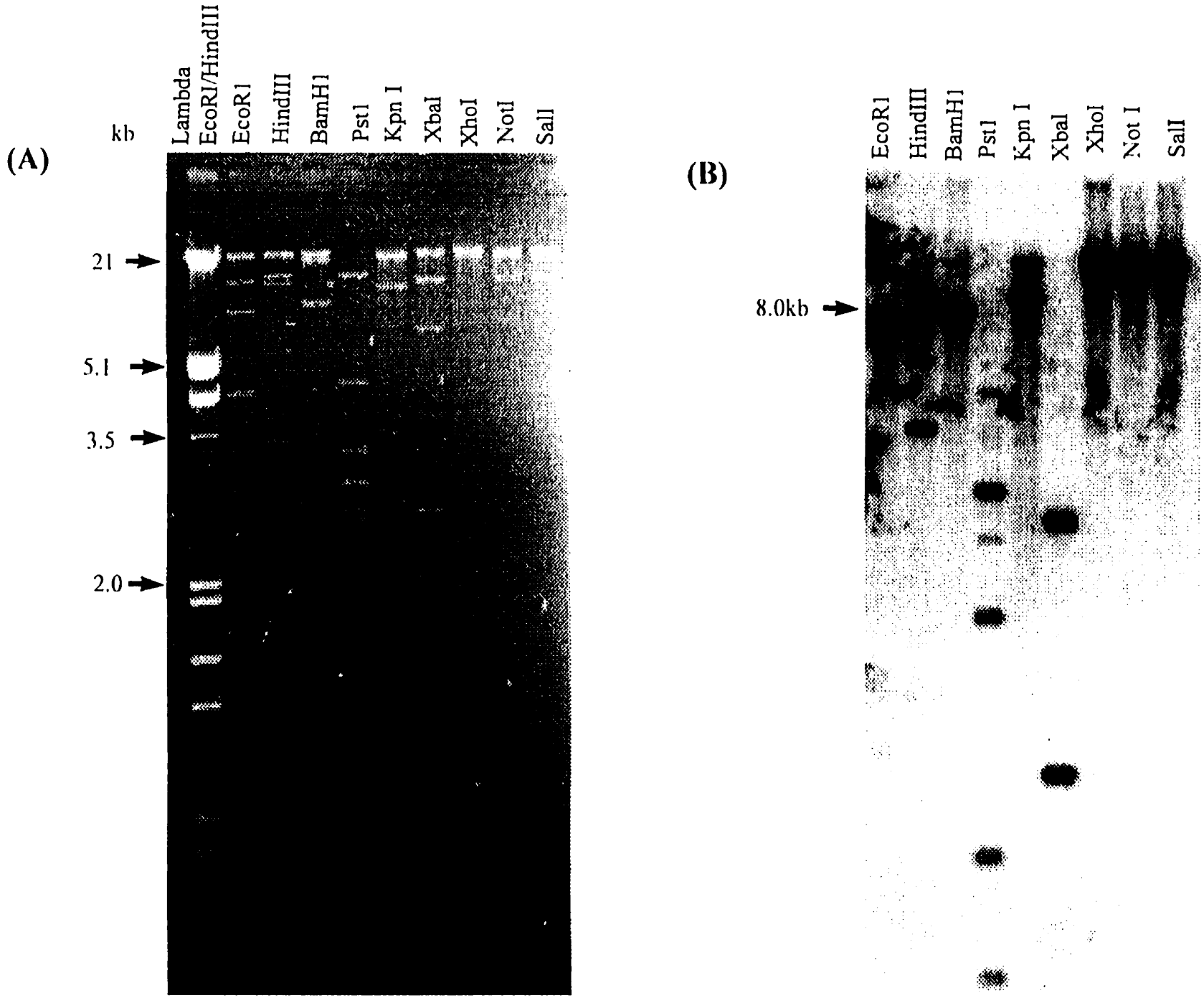
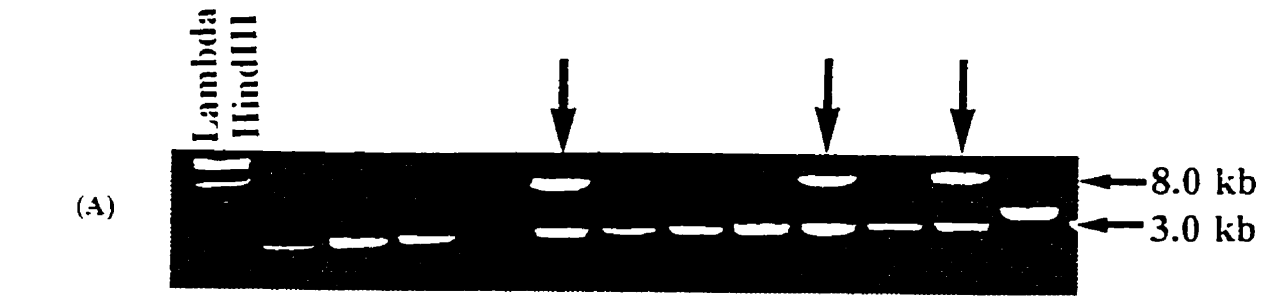
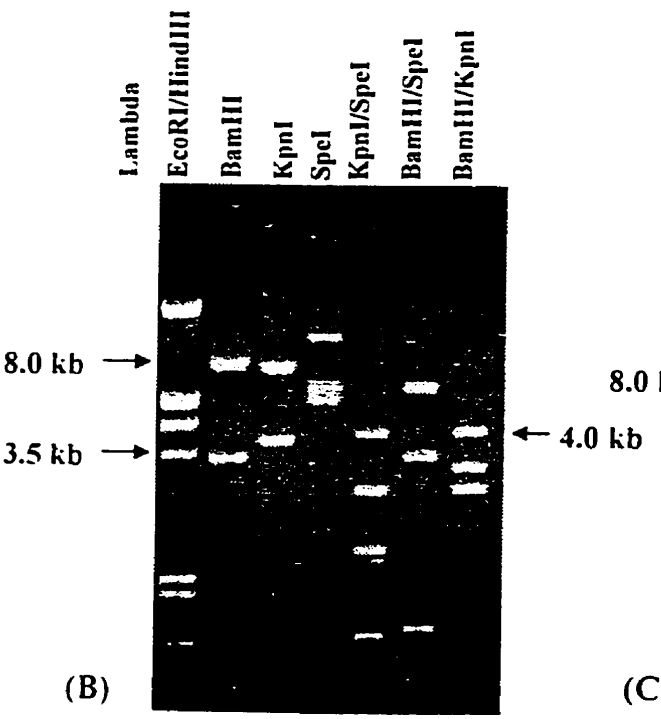

(A)
kb
Lambda EcoRI/HindIII
EcoR1
HindIII
BamH1
Pst1
Kpn I
Xbal
Xhol
Notl
Sall
21
5.1
3.5
2.0
(B)
EcoR1
HindIII
BamH1
Pst1
Kpn I
Xbal
Xhol
Not I
SalI
8.0kb

Figure 4. (A) Subcloning of the BamHI fragment into p Bluescript SK. The DNA prepared from ampicillin-resistant colonies using the mini-prep method were digested with BamHI and separated by electrophoresis in a 1.0 % of agarose gel. A 8.0 kb of BamHI and 3.0 kb of vector can be seen on the gel as indicated by the arrows. (B) Digestion of BamHI fragments in pBluescript SK. (C) Corresponding Southern blot analysis hybridization of BamHI fragment in p Bluescript SK. The BamHI fragment subcloned in p Bluescript SK were digested with different enzymes as shown. The DNA was electrophoresed through a 1.0 % agarose gel and transferred to a nitrocellulose membrane, hybridized with a ^{32}P labeled restriction fragment containing 3' sequence of exon1, exon2-3, and exon 4 of human IGFBP-3 (referring to the map of human IGFBP-3 in Figure 1). Autoradiography was carried out for 2 hr. The positions of the DNA size markers, HindIII-EcoRI digested bacteriophage λ DNA, are also shown.


Lambda
HindIII
(A)
8.0 kb
3.0 kb


Lambda
EcoRI/HindIII
BamHI
KpnI
SpeI
KpnI/SpeI
BamHI/SpeI
BamHI/KpnI
8.0 kb
3.5 kb
4.0 kb
(B)

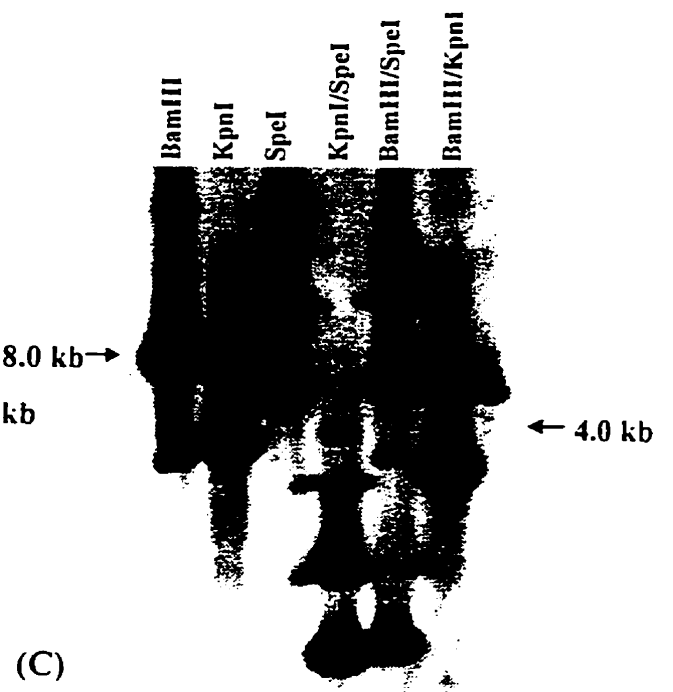
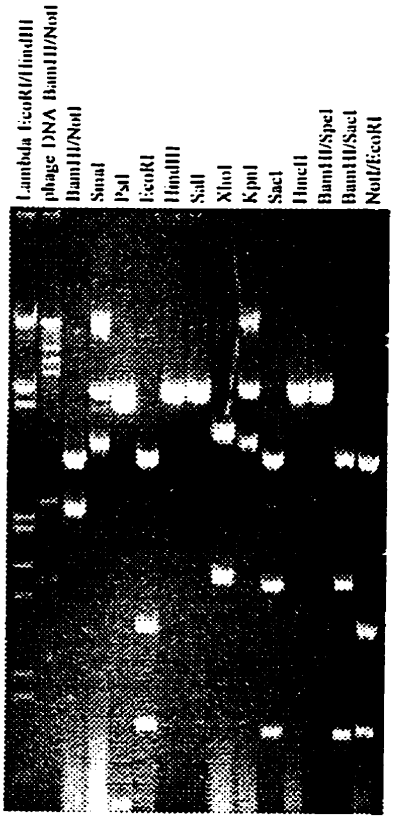

BamHI
KpnI
SpeI
KpnI/SpeI
BamHI/SpeI
BamHI/KpnI
8.0 kb
4.0 kb
(C)

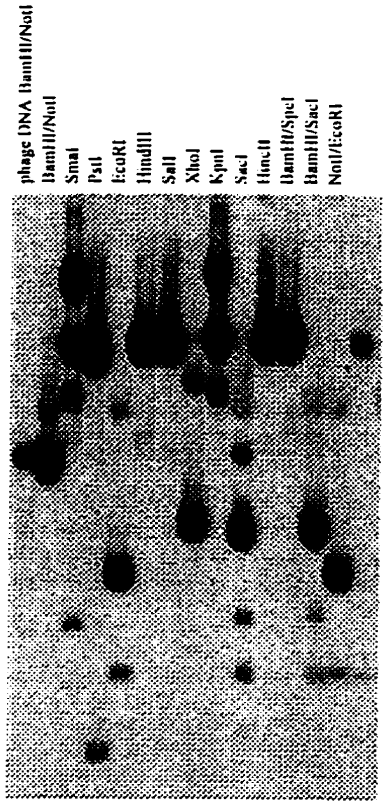
Figure 5. Identification of 5' fragment of the mouse IGFBP-3 gene. The phage DNA containing the mouse IGFBP-3 was digested with different enzymes as indicated in the Figure, separated in a 1.0 % agarose gel, transferred to a nitrocellulose membrane, and hybridized with SacII fragment which contains sequences of exon 1 of human IGFBP-3 (referred to the map in Figure 1). Autoradiography was performed for 48h. (A) Ethidium bromide stained gel of DNA containing the mouse IGFBP-3. (B) Southern blot hybridization. A BamHI/NotI fragment which was used as 5' fragment for the targeting vector was marked by a arrow and subcloned into p Bluescript SK.

Lambda
EcoRI/HindIII
PstI
BamHI/PstI
XhoI
BamHI/XhoI
NotI
BamHI/NotI
NotI/XhoI
BamHI/XbaI
SacI
BamhI/SacI
2.2 kb
(A)
PstI
BamHI/PstI
XhoI
BamHI/XhoI
NotI
BamHI/NotI
NotI/XhoI
BamHI/XbaI
SacI
BamHI/SacI
2.2 kb
(B)

Figure 6. Analysis of 5' fragment of the mouse IGFBP-3. DNA digestion, electrophoresis, transfer and hybridization were performed as before. (A) BamHI/NotI fragment subcloned in p Bluescrit SK was digested with the endonuclease restriction enzymes as indicated in the figure. (B) Corresponding Southern blot hybridization analysis using SacII fragment as a probe. Only two bands can be seen in the XhoI lane.


Lambda EcoRI/HindIII
phage DNA BamHI/NotI
BamHI/NotI
SmaI
PstI
EcoRI
HindIII
SalI
XhoI
KpnI
SacI
HincII
BamHI/SpeI
BamHI/SacI
NotI/EcoRI

(A)


phage DNA BamHI/NotI
BamHI/NotI
SmaI
PstI
EcoRI
HindIII
SalI
XhoI
KpnI
SacI
HincII
BamHI/SpeI
BamHI/SacI
NotI/EcoRI

(B)

Figure 7. (A) Southern blot hybridization analysis of BamHI-KpnI and KpnI fragments. Probe: PstI fragment containing exon-5 of human IGFBP-3 (position: +1813-+2294, referred to Figure 1) labeled with ^{32}P. Only BamHI-KpnI fragment hybridized with PstI fragment. (B) Phage DNA containing the mouse IGFBP-3 gene was digested with enzymes as shown in the figure and hybridized with SacII fragment (see Figure 1). A 6.2 kb of XhoI/KpnI fragment showed a good signal in hybridizing with SacII fragment which contains exon 1 of human IGFBP-3 cDNA was isolated and subcloned into p Bluescript for analysis.

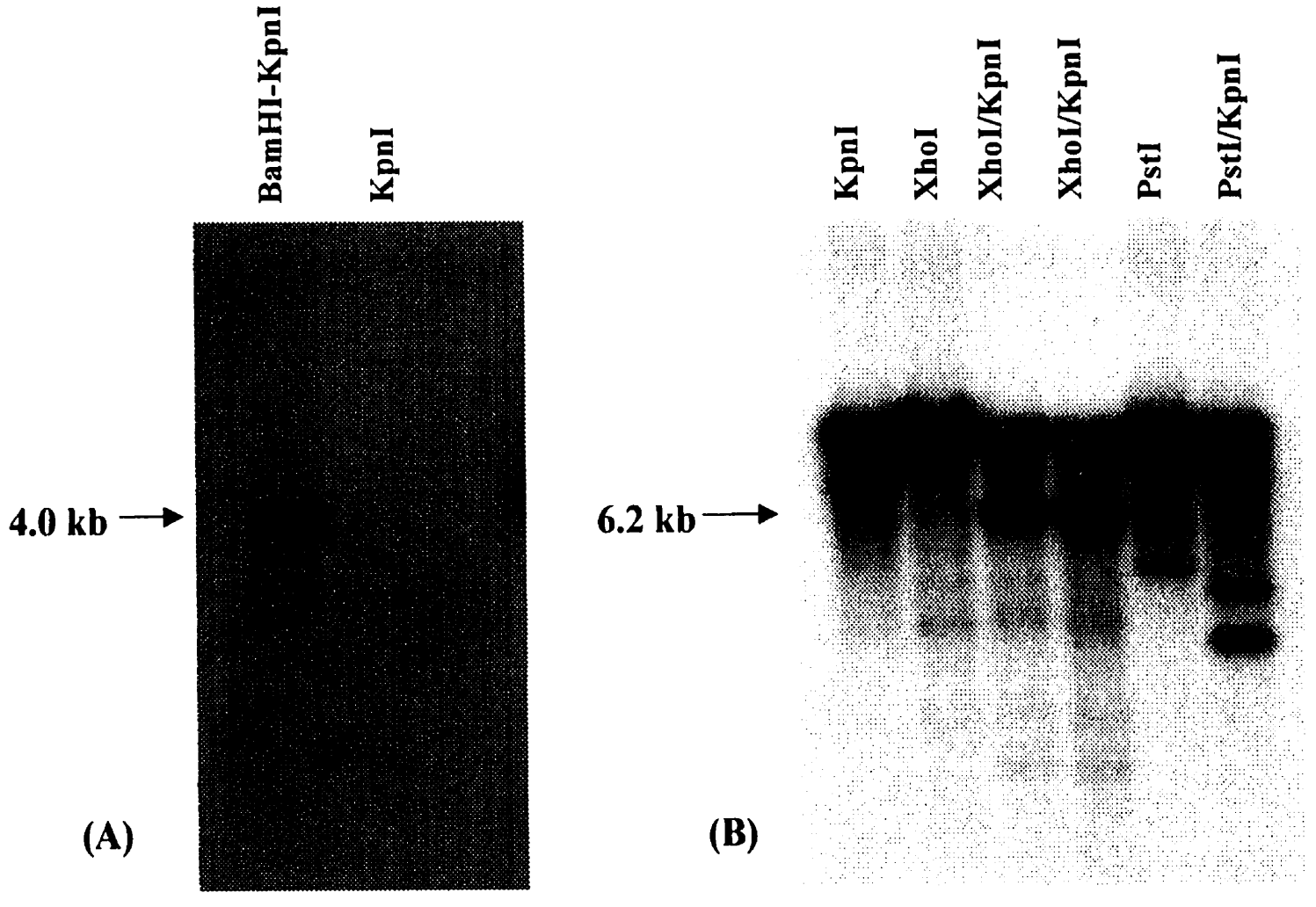
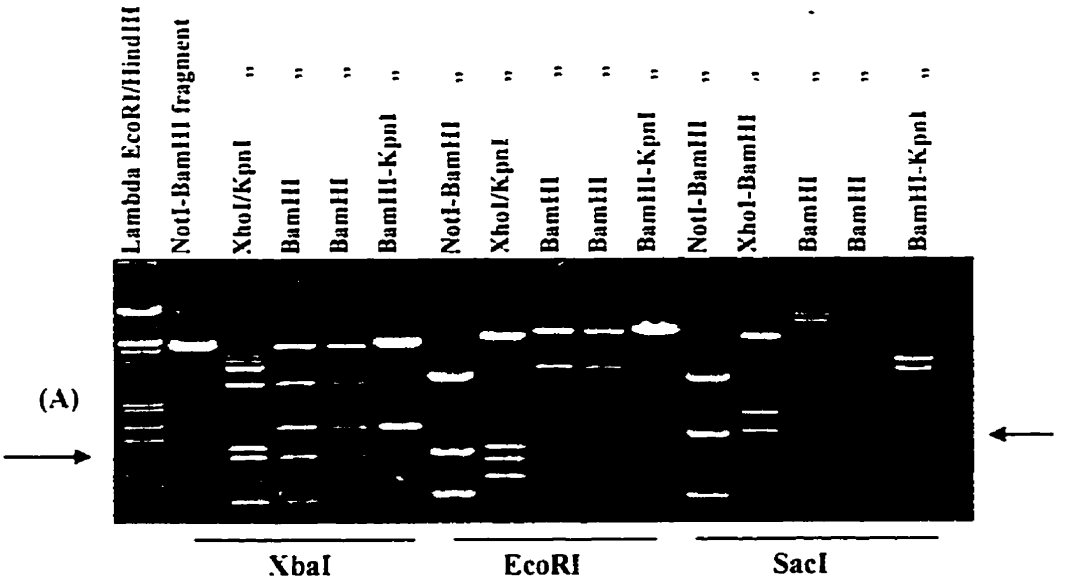

BamHI-KpnI
KpnI
4.0 kb
(A)
KpnI
XhoI
XhoI/KpnI
XhoI/KpnI
PstI
PstI/KpnI
6.2 kb
(B)

Figure 8. Relationship between NotI/BamHI, XhoI/KpnI, BamHI, BamHI/KpnI fragments. These four fragments subcloned in p Bluscript were digested together with six different enzymes and separated in 1% agarose gel. (A) and (B) are the ethidium bromide stained gel of DNA. The names of fragments digested are on the top of each gel and enzymes used are at the bottom. The common bands released from different fragments were marked by the arrows.

Relationship between NotI-BamHI,

BamHI-KpnI and XhoI-KpnI fragments from mouse IGFBP-3 gene



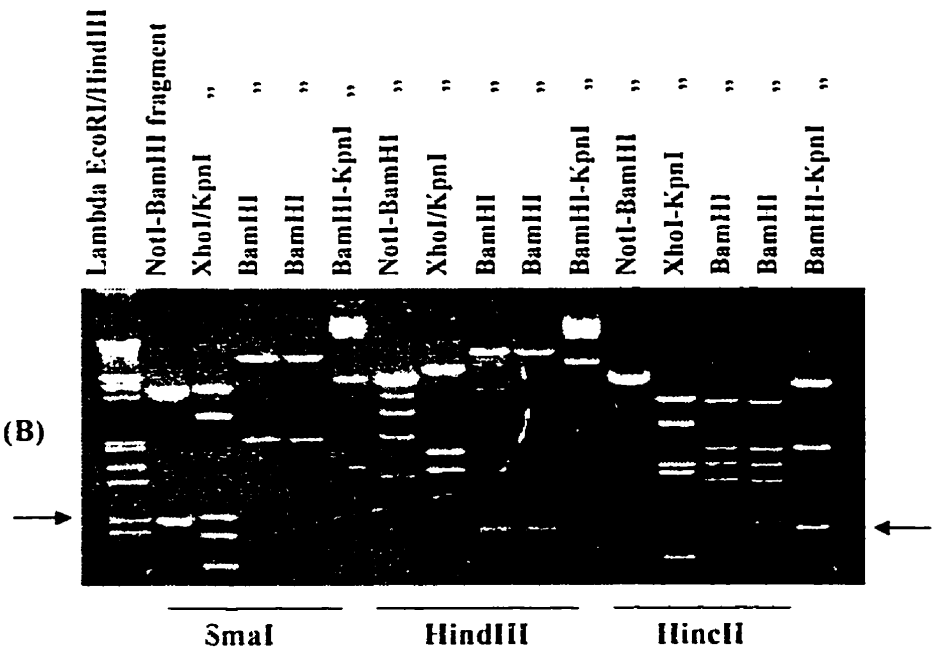
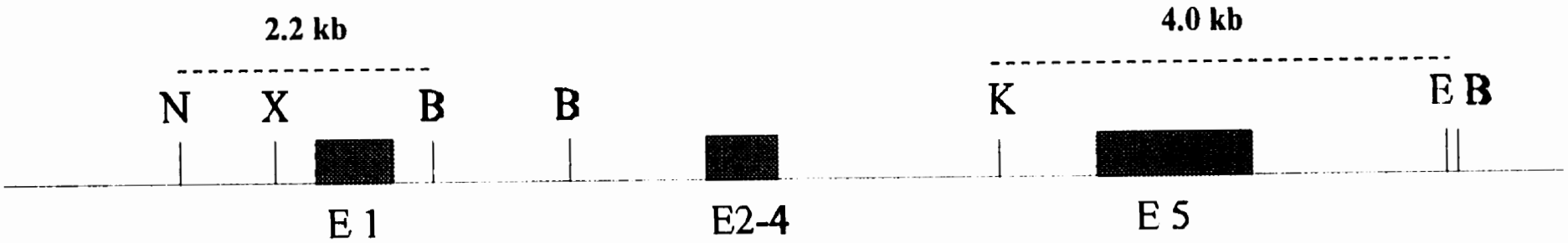


Figure 9. Schematic restriction map of mouse IGFBP-3 gene

Based on the restriction analysis of mouse IGFBP-3 gene. A restriction map of mouse IGFBP-3 was established. The filled boxes represent exons.The line between boxes are introns. Restriction sites: E=EcoRI, B=BamHI, K=KpnI, N=NotI, X=XhoI.

Mouse IGFBP-3 restriction map



The filled boxes represent exons and the lines between exons are introns

Restriction sites: E: EcoRI, B: BamHI, K: KpnI, N: NotI, X: XhoI

Figure 10. The structure of pPNT(7.2kb)

This is the vector used for constructing the targeting vector of the mouse IGFBP-3. The shaded arrows represent the mouse phosphoglycerate kinase-1 (PGK-1) promoter, the hatched boxes represent the PGK-1 poly (A) additional sequences, the open boxes are the Neo and HSV-tk genes as labeled, and the line represents the plasmid backbone. Unique restriction sites are indicated.

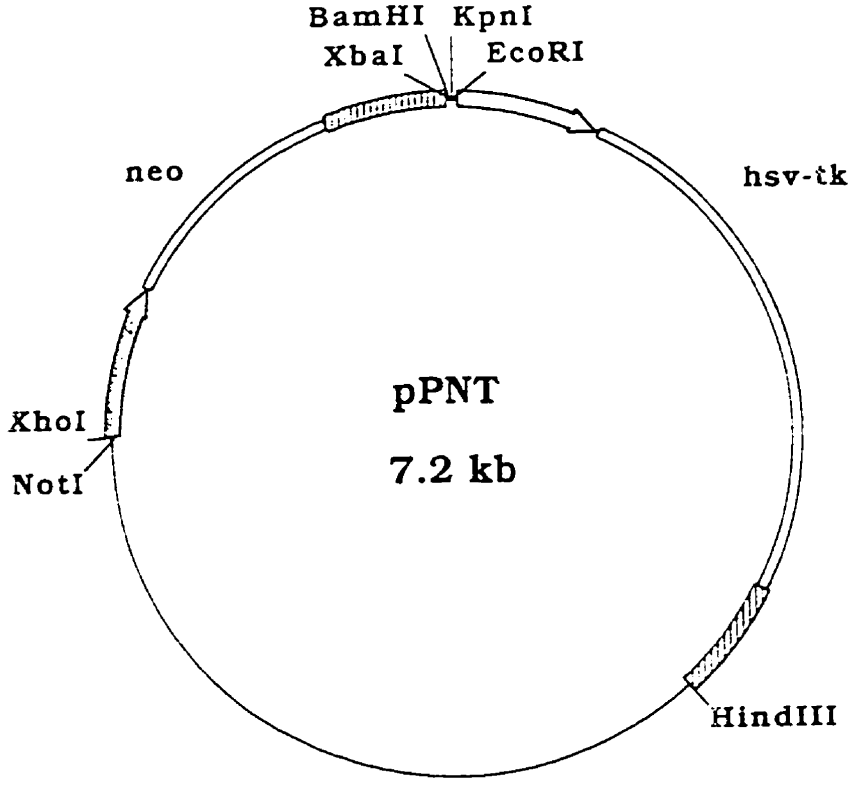

BamHI
KpnI
XbaI
EcoRI
neo
hsv-tk
pPNT
7.2 kb
XhoI
NotI
HindIII

Figure 11. Construction of targeting vector for mouse IGFBP-3. 3' fragment (KpnI/BamHI Fragment) and 5' fragment (BamHI/NotI fragment) were modified to KpnI/EcoRI and SalI/NotI respectively (see "the construction of targeting vector" in the result part) and both were subcloned into pPNT subsequently. The final targeting vector contains two homologous sequences of mouse IGFBP-3 gene, and the Neo^r is in between the two homologous sequences and the HSV-tk gene is outside of homologous region.

Construction of targeting vector

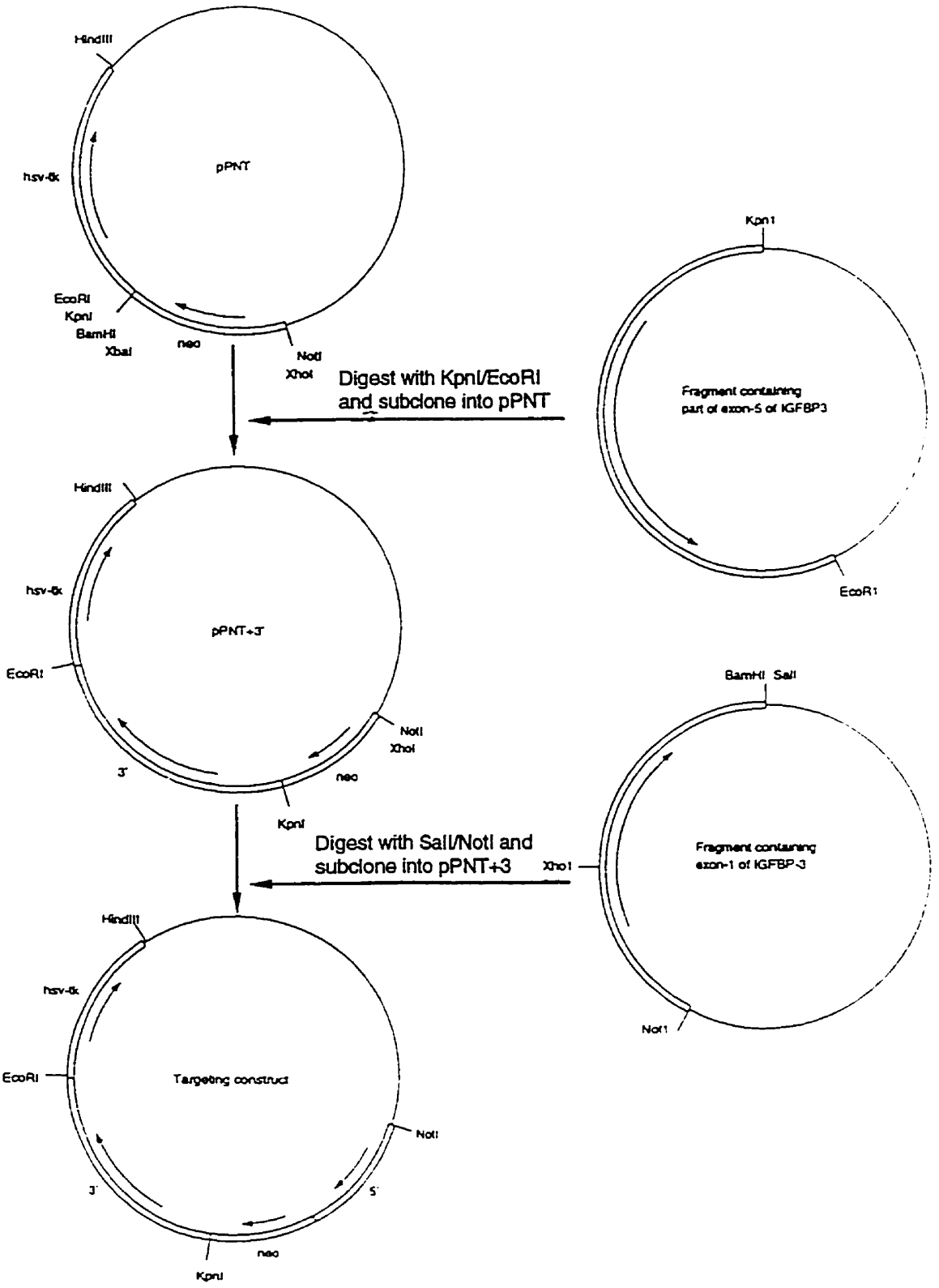


Figure 12. Confirmation of taregting vector. The targeting vector was digested together with 3' fragment SalI/NotI and 5' fragment KpnI/EcoRI and separated in 1% agarose gel. The figure shows the ethidium bromide stained gel of DNA. After digestion, the targeting vector lane gave five bands, one band was the same as the band released by 3' frgament (KpnI/EcoRI fragment); two bands were as same as the bands released by 5' fragment (BamHI/NotI fragment).

Confirmation of targeting vector

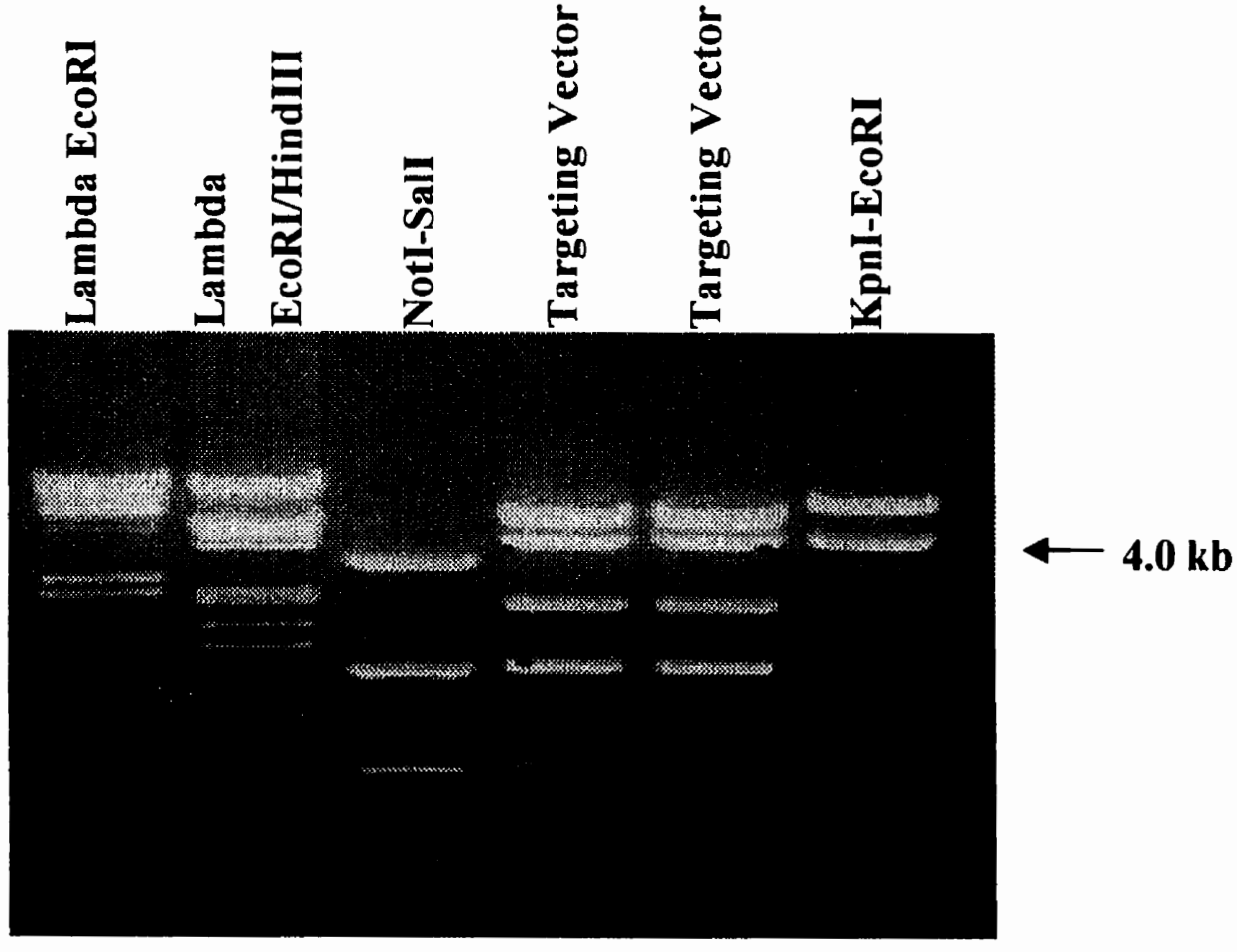
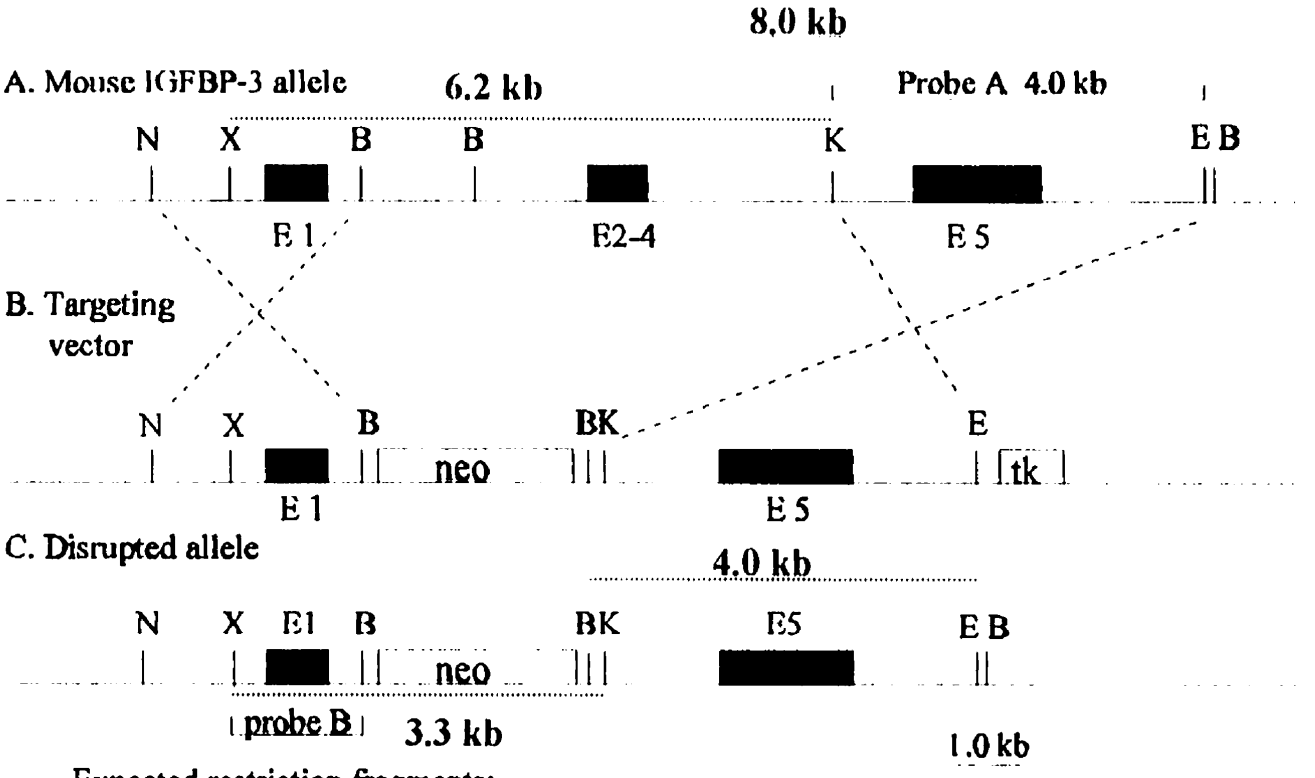


Figure 13. Targeted disruption of the mouse IGFBP-3 in ES cells

A, Restriction map of mouse IGFBP-3 allele. B, Structure of targeting vector. A neo cassette was inserted to replace exon 2-4 of mouse IGFBP-3 gene and used as a marker for positive selection. A HSV-tk cassette was placed at the end of the targeting vector for negative selection. C, The predicated structure of the disrupted allele. The probe A and B used for identification of homologous recombination are shown. The restriction sites are : BamHI (B), EcoRI (E), KpnI (K), NotI (N), XhoI (X). The black boxes represent the exons.

Disruption of the IGFBP-3 gene in mouse embryonic stem cells

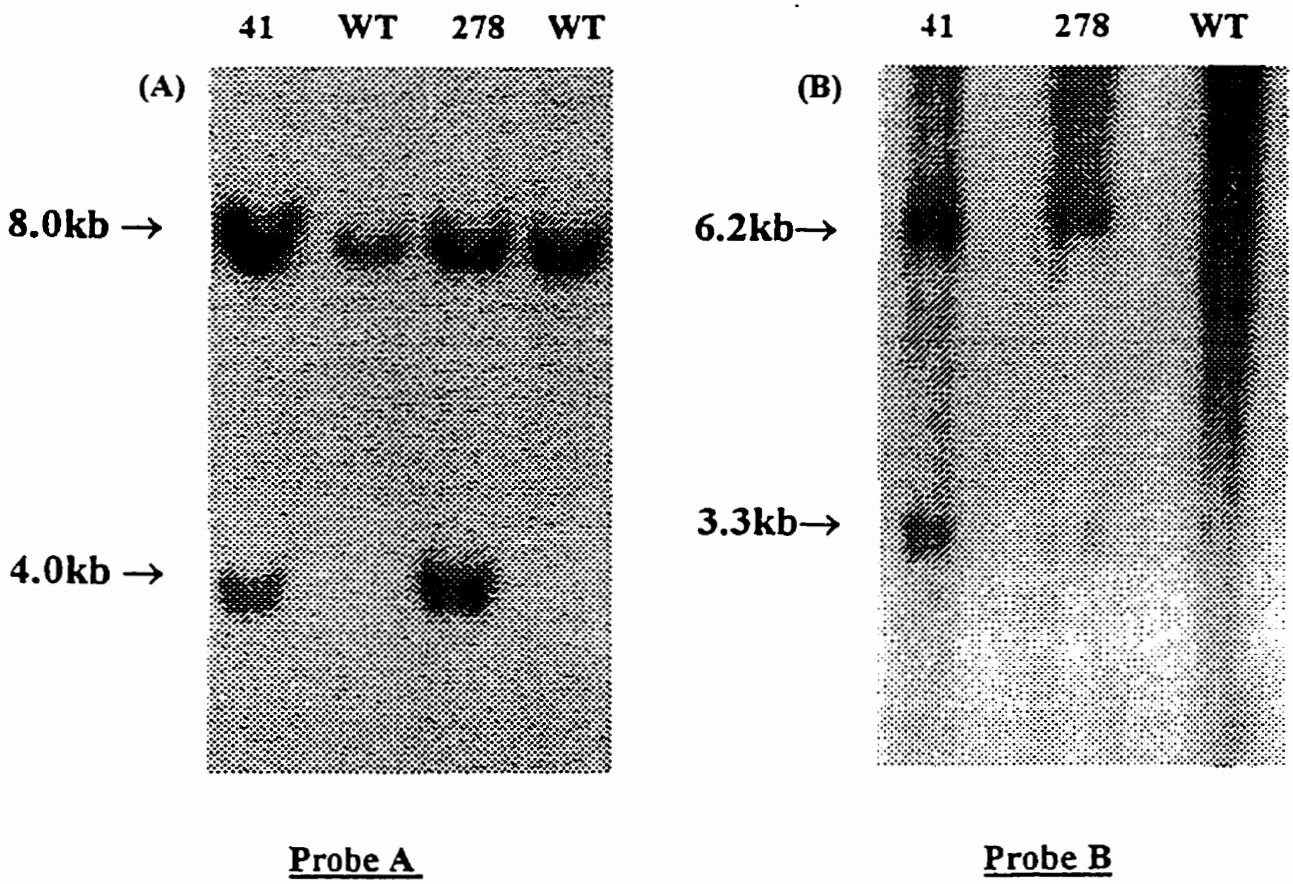


Expected restriction fragments:

	BamHI Probe A	XhoI/KpnI Probe B
Wild-type allele	8.0 kb	6.2 kb
Disrupted allele	4.0 kb	3.3 kb

Restriction sites: B:BamHI, E: EcoRI, K: KpnI, N: NotI, X: XbaII

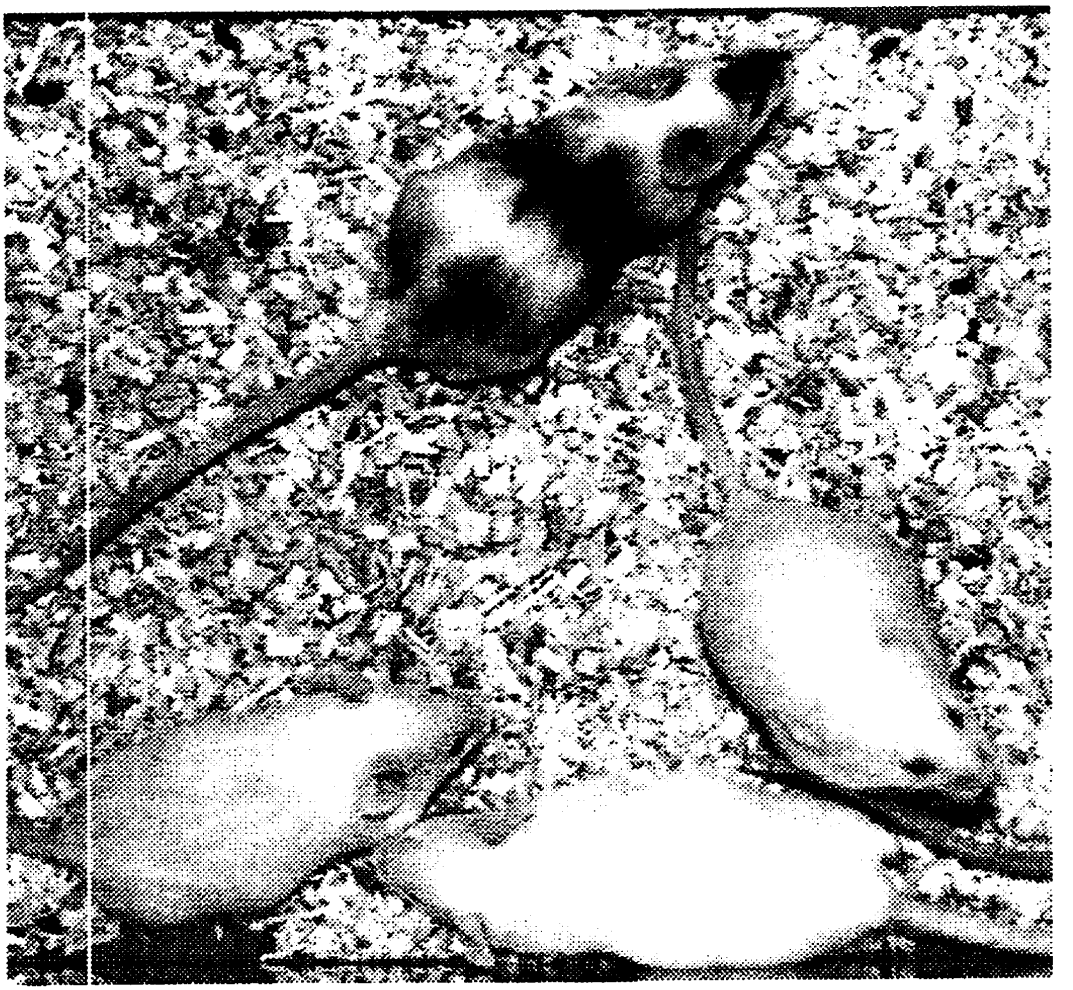
Figure 14. Southern blot hybridization analysis of genomic DNA from control ES cell (R1) and IGFBP-3 targeted clones 41 and 278. The Southern blot hybridization procedure was performed as before. In (A) the DNA was digested with BamHI and subjected to southern blot hybridization analysis using probe A according to the strategy in Figure 13. A 8.0 kb of wild type allele and 4.0 kb of mutant allele were indicated by the arrows. In (B) the DNA was digested with XhoI and KpnI, and a 6.2 kb of wild type allele and 3.3 kb of disrupted allele were shown when hybridized with probe B.



Probe A

Probe B

Figure 15. A IGFBP-3 chimeric mouse with normal CD1 mice. The chimeric mouse shows some black hair which is contributed from 41 ES cell clone (Figure 14). In contrast, the normal CD1 mice have entirely white hair.



Chapter 5. DISCUSSION

IGFBP-3 is the predominant binding protein for IGFs in circulation. It functions as a modulator of IGFs biological actions, as well as storage and transportation for IGFs in serum (Jones and Clemmons, 1995). In addition, recent studies showed that IGFBP-3 has IGF independent actions on cell growth. Investigations on IGFBP-3 in the past several years have revealed inconsistent results. This may be attributed in part to the complexity of experimental conditions. To study the physiological function of IGFBP-3, gene targeting technique was employed in this study to generate IGFBP-3 null mutation mice. Since IGFBP-3 is the major binding protein for IGFs in the circulation, the following outcome of the IGFBP-3 null model can be predicated. First the IGFBP-3 knockout mice could be phenotypically normal, this may suggest that the IGFBP-3 plays little role in the regulation of the action of IGFs or that the IGFBP-3 is important for the biological actions of IGFs, but the function of IGFBP-3 has been taken over by the other IGF binding proteins. Thus the IGFBP-3 knockout mice compensate effectively for the loss of IGFBP-3. Second, the IGFBP-3 knockout mice die as embryos, indicating that IGFBP-3 is crucial for the development of the mouse. If the IGFBP-3 mutation is embryonic lethal, the IGFBP-3 gene could be deleted in mice after birth by using a conditional knockout procedure (Gu H et al ., 1994) in which the gene of interest can be disrupted at certain stage of development or in a specific tissue. In this manner the function of IGFBP-3 can still be studied in mice where the IGFBP-3 is disrupted. Third, the IGFBP-3 null mice could survive but show abnormalities that might include alteration in body

size or metabolism, if an altered phenotype is observed in the IGFBP-3 knockout mice, this mice confirm an important role of IGFBP-3 in modulating IGFs bioactivity.

In this research, the mouse IGFBP-3 gene was successfully isolated from a mouse genomic library and a mouse IGFBP-3 gene targeting vector was developed (Figure11 & 12). The mouse IGFBP-3 gene was successfully disrupted in embryonic stem cell and 7 chimeric mice for mouse IGFBP-3 were generated by blastocyst injection.

Gene targeting in ES cells has been widely used in the study of functions of different genes. Gene targeting is achieved through a process of homologous recombination between a targeting vector and an endogenous gene. There are two types of targeting vectors, replacement and insertion vectors. The most commonly used vector is replacement vector. In my study, the replacement vector was chosen for the gene targeting. Replacement vector is intended to replace homologous gene sequences with a selectable mutation. Thus typically, replacement vector contains sequences homologous to the target gene sequences (Thomas and Capecchi, 1987). In order to disrupt mouse IGFBP-3 gene, at first the mouse IGFBP-3 gene was identified and isolated by screening a mouse genomic library (Lambda Dash 129 Library). After analysis of the mouse IGFBP-3 gene, a restriction map of the mouse IGFBP-3 was established (Figure 9). A 2.2 kb of SalI/NotI fragment from the mouse IGFBP-3 was selected as 5' fragment for the targeting vector, and a 4.0 kb of EcoRI/KpnI fragment from mouse IGFBP-3 was chosen to construct 3' fragment of the targeting vector. Both fragments were subcloned

into pPNT (see the structure in Figure 10). The advantage of using this pPNT is that the Neo^r and tk genes have already engineered in the plasmid backbone. Once the two homologous sequences have been put into this vector, the construction of targeting is completed. The targeting vector of the mouse IGFBP-3 gene has met the requirement for the construction of the replacement vector (Joyner, 1993). Although different lengths of homologous sequences have been reported in gene targeting. The most commonly used length of homologous sequences is 5-8 kb. Too long of homologous sequence is considered to increase chances of nonhomologous recombination. In this study, the homologous sequences is 6.2 kb, and an average of 0.36% homologous recombination was obtained (Table 2).

The primary importance of ES cell culture is to maintain its undifferentiated state so that it is able to contribute to all of the cell lineages including germ-line. ES cells are generally cultured on a feeder layer of embryonic fibroblasts or in the presence of leukemia inhibitory factor (LIF) to prevent differentiation. Williams et al (1988) demonstrated that ES cells can be maintained in the medium supplemented with LIF up to 22 passages (approximately 100 cell generations), without changes of the growth characteristics of the these cells, and are capable of giving rise to germ line chimeric mice which is the most important evidence of pluripotency of ES cells. Under most circumstance of this study, ES cells were cultured in the presence of LIF at the concentration of 1000 units ml^{-1}, and passaged exactly every two days. 90% of ES cells displayed undifferentiated stem cell phenotype under this condition, characterized by

compact colonies of small cells with a large nuclear to cytoplasmic ratio, and retained the ability to produce chimeric mice when injected into CD1 blastocysts (Table 2 and Figure 15). It suggested that ES cells can be maintained in the presence of LIF instead of feeder layers. In the late stage of this study, both the feeder layer (primary embryonic fibroblast cell) and LIF were used in the culture of ES cells. However, under this condition, the ES cells did not show morphological difference from the ES cells in LIF or their ability to give rise to chimeric mice. These conditions were used for short time and only one chimera was produced from the ES cells maintained on the feeder layer. More experiments are needed to test the culture conditions for this ES cell line (R1) in order to create highly chimeric mice.

Positive-negative selection has now almost become the standard ES cell screening procedure in gene targeting. In the current investigation, positive-negative selection was applied to the ES cells 48hrs after electroporation. G418 was used for positive selection where only the ES cells expressing Neo^r could survive, whereas Gancyclovir for negative selection against the ES cells expressing HSV-tk gene. The dose of G418 used was between 150 and 250μg/ml, and gancyclovir was used at the final concentration of 2nM. With this concentrations of G418 and gancyclovir, an average of 422 colonies was obtained from $1.5x10^7$ ES cell which survived electroporation (Table 1). Of the 550 colonies screened by Southern blot hybridization analysis, two was identified to carry homologous recombination in the mouse IGFBP-3 locus (Figure 14). The average targeting frequency in this study is 0.8%, which is lower than most of reports in recent

years. The targeting frequency is highly variable among the reports. For example, Mansour (1988) reported 5% of homologous recombination and one report was 25% (Paszty et al., 1995). The homologous recombination frequency may depend upon many factors such as length of sequence homology, chromosomal location, and transcriptional state of the gene under study. In the investigation conducted by Paszty (1995), homologous sequence used for targeting vector was 45 kb, much longer than the average length. It is difficult to determine the reasons of low homologous recombination frequency in this study. One factor influencing the low homologous recombination frequency may be the expression of Neo^r and tk, particularly tk. In this study, wild type ES cells were tested with G418. The test showed ES cells died after the treatment of G418. It is difficult to determine how much of tk is expressed after the targeting vector is introduced into ES cells. If the expression of tk is not sufficient, or if tk is not expressed, then one can assume that negative selection efficiency will be low, which will definitely decrease screening efficiency.

There are two ways of generating mutant gene mice, blastocyst microinjection and aggregation. The predominant method used so far has been the blastocyst microinjection. In this study, blastocyst injection was utilized to generate mouse IGFBP-3 null mutation mice after mouse IGFBP-3 gene was successfully disrupted in ES cells (Figure 14). So far seven chimeric mice have been generated by blastocyst injection, the range of chimerism was 30%-50% (Tables 3, 4). Of 7 chimeric mice generated, none has been found to carry germ line transmission. Apparently low chimerism accounts for the low

frequency of germ-line transmission, since the chimeric level in the chimeric mice parallels with the germ-line transmission, therefore highly chimeric mice of mouse IGFBP-3 need to be generated in order to obtain germ-line transmission in the future. CD1 strain of mouse was used as host embryos in this study. It was reported that CD1 produced poor chimera (Joyner, 1993), and the result of chimera production in this study is consistent with the report. Other blastocyst strains which have been approved producing good chimera should be used. The pluripotency of the ES cell is most crucial in the formation of chimera. Under most circumstances in this study, the ES cell (R1) showed undifferentiated stem cell phenotype, such as compact colonies of small cells with a large nuclear to cytoplasmic ratio. However, the production of chimeras from this ES cell line in this study is very low compared with other reportes.This may suggest that the pluripotency of ES cell used in this study was decreased. New fresh ES cell should be used and the pluripotency of the ES cell can be tested by producing highly chimeric mice to make sure that the ES cell is fully pluripotent.

REFERENCES

Albiston AL, and Herington AC 1990 Cloning and characterization of the growth hormone-dependent insulin-like growth factor binding protein (IGFBP-3) in the rat. Biochem Biophys Res Commun. 166:892-897.

Andress DL, Birnbaum RS 1992 Human osteoblast-derived insulin-like growth factor (IGF) binding protein-5 stimulates osteoblast mitogenesis and potentiates IGF action. J Biol Chem 267:22467-22472.

Argente J, Barrios V, Pozo J, Munoz MT, Hervas F, Stene M, Hernandes M 1993 Normative data for insulin-like growth factors (IGFs), IGF binding proteins, and growth hormone binding protein in a healthy Spanish pediatric population: age- and sex-related changes. J Clin Endocrinology Metab77: 1522-1528

Batch JA, Baxter RC, Werther GA 1991 abnormal regulation of insulin-like growth factor binding proteins in adolescents with insulin-dependent diabetes. J Clin Endocrinol Metab 73:964-968.

Baxter RC, Martin JL 1986 Radioimmunoassay of growth hormone-dependent insulin-like growth factor protein in human plasma. J Clin Invest 78:1504-1512.

Baxter RC, Martin JI, Tyler MI, Howden MEH.1986 Growth hormone-dependent insulin-like growth factor (IGF) binding protein from human plasma differs from other human IGF binding proteins. Biochem Biophys Res Commun. 139:1256-1261.

Baxter RC, Martin JI 1989 Binding proteins for the insulin-like growth factors: structure, regulation and function. Prog Growth Factor Res 1:49-68

Bereket A, Lang CH, Blethen SL, Gelato MC, Fan J, Frost RA, Wilson TA 1995 Effect of insulin on the insulin-like growth factor system in children with new-onset insulin-dependent diabetes mellitus. J Clin Endocrinol Metab 80(4): 1312-1317

Bereket A, Lang CH, Blethen SL, Fan J, Frost RA, Wilson TA 1995 Insulin-like growth factor binding protein-3 proteolysis in children with insulin-dependent diabetes mellitus: a possible role for insulin in the regulation of IGFBP-3 protease activity. J Clin Endocrinol Metab 80:2282-2288.

Betty C, Villafuerte, Wei-Ning Zhang, and Lawrence S. Philips 1996 Insulin-like growth factor-I regulates hepatic insulin-like growth factor binding protein-3 by different mechanisms. Molecular Endocrinology 10: 622-630

Bicsak TA, Shimonaka M, Malkowski M, Ling N 1990 Insulin-like growth factor-binding protein (IGF-BP) inhibition of granulosa cell function:effect of cyclic adenosine 3', 5'-

monophosphate, deoxyribonucleic acid synthesis, and comparison with the effect of an IGF-I antibody. Endocrinology 126:2184-2189.

Binoux M, Lalou C, Lassarre C, Segovia B 1994 Regulation of IGF availability by IGFBP proteases. in: Baxter RC, Gluckman PD, Rosefeld RG(eds) The insulin-like growth factors and their regulatory proteins. Elsevier Science. Amsterdam, pp 217-226.

Blat C, Delbe J, Villaudy J, Chatelain PG, Golde A, Harel L 1989 Isolation and amino-terminal sequence of a novel cellular growth inhibitor (inhibitory diffusible factor 45) secreted by 3T3 fibroblasts . J Biol Chem 264: 6021-6024

Blum WF, Jenne EW, Reppin F, Kietzmann K, Ranke MB, Bierich JR 1989 Insulin-like growth factor (IGF) binding protein complex is a better mitogen than free IGF-I Endocrinology 125:766-772.

Blum WF, Horn N, Kratzsch J, Jorgensen JO, Juul A, Teale D, Mohnike K, Ranke MB 1993 Clinical studies of IGFBP-2 by radioimmunoassay. Growth Regul 3:100-104

Blum WF, Ranke MB, Kietzmann K, Gauggel E, Zeisel HJ, Beierich JR 1990 A specific radioimmunoassay for the growth (GH)-dependent somatomedin binding protein:its use for diagnosis of GH deficiency. J Cinl Endicrinol Metab 70: 1292-1298

Brinkamn A, Groffen CA, Kortler DJ, Drop SLS. 1988 Organization of the gene encoding the insulin-like growth factor binding protein IBP-I. Biochem Biophys Res Commun 157:898-907

Clark RG, Mortensen D, Reifsynder D, Mohler M, Etcheverry T, Mukku V 1993 Recombinant human insulin-like growth factor binding protein-3 (rhIGFBP-3): effects on the glycemic and growth promoting activities of rhIGF-I in the rat. Growth Regul 3:50-52

Clemmons DR 1992 IGF Binding proteins: regulation of celluar actions. Growth Regulation 2, 80-87

Cohen P, Lamson G, Okajima T, Rosenfeld GR 1993 Transfection of the human IGFBP-3 gene into Balb/c fibroblast: a model for the cellular function of IGFBPs. Growth Regul (1): 23-26

Cohen P, Peehl DM, Stamey TA, Wilson KF, Clemmons DR, Rosenfeld RG 1993 Elevated levels of insulin-like growth factor protein-2 in the serum of prostate cancer patients. J Clin Endocrinol Metab 76:1031-1035.

Cohick WS, Clemmons DR 1993 The insin-like growth factors: Annu Rev Physiol 55:131-153

Cornell HJ, Embery G, Herington AC 1986 Association of insulin-like growth factors with metabolically inactive and active carrier bound complexes in sera. J Endocrinol 241:744-749

Conover CA 1990 Regulation of insulin-like growth factor (IGF) binding protein synthesis by insulin and IGF-I in cultured bovine fibroblasts. Endocrinology 26:3139-3145

Conover CA, Ronk M, Lombana F, Powell DR 1990 Structural and biological characterization of bovine insulin-like growth factor binding protein-3. Endocrinology 127:2795-2803.

Conover CA, Powell DR 1991 Insulin-like growth factor (IGF) binding protein-3 blocks IGF-I induced receptor down-regulation and cell densitization in cultured bovine fibroblasts. Endocrinology 129:710-716

Concver CA 1991 A unique receptor-independent mechanism by which insulin-like factor I regulates the availability of insulin-like growth factor proteins in normal and transformed human fibroblasts. J Clin Invest 88:1354-1361

Cotterill AM, Holly JMP, Taylor AM, Davis SC, Coulson VJ, preece MA, WassJA, Savage MO 1992 The insulin-like growth factor (IGF)-binding proteins and IGF bioactivity in Laron-type dwarfism. J Clin Endocrinol Metab 74:56-63

Cubbage ML, Suwanichkul A, Powell DR. Insulin-like growth factor binding protein-3: Organization of the human chromosomal gene and demonstration of promoter activity. J Biol Chem. 1990; 265:12642-12649.

Davenport ML, Clemmon DR, Milles MV, Camacho-Hubner C, D'ErcoleAJ, Underwood LE 1990 Regulation of serum insulin-like growth factor-I (IGF-I) and IGF binding proteins during rat pregnancy. Endocrinology 127:1278-1286

DeMellow JSM, Baxter RC 1988 Growth hormone dependent insulin-like growth factor binding protein both inhibits and potentiates IGF-I stimulated DNA synthesis in skin fibroblasts. Biochem.Biophys Res Commun. 156:199-204.

Donahue LR, Hunter SJ, Sherblom AP, Rosen CJ 1990 Age-related changes in serum insulin-like growth factor binding protein in women. J Clin Endocrinol Metab 71:575-579

Dugaiczyk A, Boyer H, Goodman H 1975 Lagation of EcoRI endonuclease generated DNA fragments into liner and circular structure. J Mol Biol.96:174-184.

Ehrenborg E, Vilhelmsdotter S, Bajalica S, Larsson C, Stern I, Koch J, Brondum-Nielsen K, Luthman H. 1991 Structural and localization of the human insulin-growth factor-binding protein 2 gene. Biochem Biophys Res Commun 176:1250-1255.

Evans MJ and Kaufamn MH 1981 Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154-156.

Feinberg A, Vogelstein B 1983 A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem. 132:6-13.

Gargosky SE, Owens PC, Walton PE, Owens JA, Robinson JS, Wallace JC, Ballard FJ 1991 Most of the circulating insulin-like growth factor-I and -II are present in the 150kDa complex during human pregnancy. J Endocrinol 131:491-497

Gibson JM, Westwood M, crosby SR, Gordon C, Holly JMP, Fraser W, Anderson C, White A, Young RJ 1995 Choice of treatment affects plasma insulin-like growth factor binding protein-I in non-insulin-like dependent diabetes mellitus. J Clin Endocrinol Metab 80:1369-1375.

Gu H, Marth JD, Orban PC, Mossmann H, Rajewsky K 1994 Deletion of a DNA Polymerase β gene Segment in T cells UsingCell Type-Specific Gene Targeting. Science 265:103-106

Guidice LC, Farreell EM, Pham H, Lamson G, Rosenfeld RG 1990 Insulin-like growth factor binding proteins in maternal serum throughout gestation and in the puerperium: Effects of a pregnancy-associated serum protease activity. J clin Endocrinol Metab 71:806-

Hardouin S, Gourmelen M, Noguiez P, Seurin D, Roghani M, Lebouc Y, Povoa G, Merimee TJ, Hossenlopp P, Binoux M 1989 Molecular forms of serum insulin-like growth factor (IGF)-binding proteins in man: relationship with growth hormone and IGFs and physiological significance. J Clin Endocrinol Metab 69:1291-1301

Hall K, Lundin G, Povoa G 1988 Serum levels of low molecular weight form of insulin-like growth factor binding protein in healthy subjects and patients with growth hormone deficiency, acromegaly and anorexia. Acta Endocrinol (Copenh) 118:321-326

Hasegawa Y, Hasegawa T, Koni H, Aso T, Kotoh S, Yamada M,Tsuchiya Y 1995 Proteolytic activity of IGFBP-3 in various clinical conditions during childhood studied by means of western immunoblotting. Endocr J 42:569-576

Johnson R, Sheng M, Greenberg M, Kolodner R, Papaioannou V, Spiegelman B 1989 Targeting of nonexpressed genes in embryonic stem cells via homologous recombination. Science 245: 1234-1236

Hossenlopp P, Binoux M 1989 Molecular forms of serum insulin-like growth factor (IGF)-binding protein in man: relationship with growth hormone and IGFs and physiological significance. J Clin Endocrinol Metab 65:432-440

Hossenlopp P, Segovia B, Lassarre C, Roghani M, Bredon M, Binoux M. 1990 Evidence of enzymatic degradation of insulin-like growth factor-binding proteins in the 150k complex during pregnancy. J Clin Endocrinol Metab. 71:797-805.

Jorgensen JOL, Blum WF, Moller N, Ranke MB, Christiansen JS 1991 Short-term changes in serum insulin-like growth factor (IGF) and IGF bing protein3 after different modes of intravenous growth hormone (GH) exposure in GH deficient patients. J Clin Endocrinol Metab 72: 582-587.

Joyner AL 1993 Gene targeting: A Practical Approach. Oxford: Oxford University Press: 1993.

Juul A, Dalgaard P, Blum WF, Bang P, Hall K, Michaelsen KF, Muller J, Skakkebaek NE 1995 Serum levels of insulin-like growth factor(IGF)-binding protein-3 (IGFBP-3) in healthy infants, children, and adolescents: the relation to IGF-I, IGF-II, IGFBP-1, IGFBP-2, age, sex, body mass index, and pubertal maturation. J Clin Endocrinol Metab 80:2534-2542.

Jones JI, Clemmons DR 1995 insulin-like growth factors and their binding proteins: biological actions. Endocr Rev 16:3-34

Juul A, Main KM, Blum W, Lindholm E, Ranke MB, Skakebaek NE 1995 The ratio between serum levels of insulin-like growth factor (IGF)-I and IGF binding proteins (IGFBP-1, IGFBP-2 and -3) decreases with age in healthy adults and is increased in acromegalic patients. Clin Endocrinol (oxf) 41:85-93.

Kanety H, Karasik A, Klinger B, silbergeld A, laron Z 1993 Long term treatment of Laron type dwarfs with insulin-like growth factor-I increases serum insulin-like growth factor binding protein-3 in the absence of growth hormone activity. Acta Endocrinol (Copench) 128: 144-149

Kanety H, Madjar Y, Dadgan Y, Levi J, Papa MZ, Pariente C, Goldwasser B, Karasik A 1993 Serum insulin-like growth factor binding protein-2 (IGFBP-2) is increased and IGFBP-3 is decreased in patients with prostatic cancer: correlation with serum prostate specific antigen. J Clin Endocrinol Metab 77:229-233

Lalou C, Lassarre C, Binoux M 1996 A proteolytic fragment of insulin-like growth factor (IGF) binding protein-3 that fails tobind IGFs inhibits the mitogenic effects of IGF-I and insulin. Endocrinology137:3206-3212.

Lamson G, Giudice LC, Cohen P, Liu F, Gargosky S, Muller HL, Oh Y, Wilson KF, Hintz RL, Rosenfeld RG 1993 Proteolysis of IGFBP-3 may be a common regulatory mechanism of IGF action in vivo. Growth Regul 3:91-95

Lee DY, Park SK, Yorgin PD, Cohen P, Oh Y, Roenfeld RG 1994 Alteration in insulin-like growth factor-binding proteins (IGFBPs) and IGFBP protease activity. J Endocrinol Metab 79:1376-1382

Luo JM and Murphy LJ 1990 Regulation of insulin-like growth factor binding protein-3 expression by dexamethasone. Mol-Cell-Endocrinol. Dec 21; 74(3):213-9

Mansour SL, Thomas KR, Capecchi MR 1988 Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cell: a general strategy for targeting mutations to non-selectable genes.Nature 336:348-352.

Mansour SL, Thomas KR, Capecchi MR 1988 Disruption mutagenesis by gene targeting in mouse-embryo-derived stem cells. Cell 5: 503-505

Maniatis T, Feitsch EF, Sambrook J 1984 Molecular Cloning: a laboratory manual.

Martin GR 1981 Establishment of pluripotential cell lines from embryos cultured in medium conditioned by teratocracinoma stem cell. Proc.Natl.Acdd.Sci.78:7634-7638

McCusker RH, Camacho-Hubner C, Bayne ML, Cascierri MA, Clemmon DR 1990 Insulin-like growth (IGF) binding to human fibroblast and glioblastoma cells: the modulating effect of cell released IGF binding proteins (IGFBPs). J cell Physiol 144:244-

253.

McCusker RH, Busby WH, Dehoff MH, Camacho-Hubner C, Clemmon DR 1991 insulin-like factor binding to cell monolayers is directly modulated by the addition of IGF-binding proteins. Endocrinology 129:939-949

Miell JP, Taylor AM, Jones J, Holly JM, Gaillard RC, Pralong FP, Ross RJ, Blum WF 1993 The effects of dexamethasone treatment on immunoreactive and bioactive insulin-like growth factors (IGFs) and IGF binding proteins in normal male volunteers J Endocrinol 136:525-533

Miell JP, Buchanan CR, Norman MR, Maheshwari HG, Blum WF 1994 The evolution of changes in immunoreactive serum insulin-like growth factors (IGFs), IGF binding proteins, circulating growth hormone (GH) and GH-binding protein as a result of short-term dexamethasone treatment. J Endocrinol 142:547-554

Muller HL, Oh Y, Gargosky SE, Lehrnbecher T, Hintz RL, Rosenfeld RG 1993 Concentrations of insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3), IGF, and IGFBP-3 protease activity in cerebrospinal fluid of children with leukemia, central nervous system tumor, or meningitis. J Clin Endocrinol Metab 77:1113-1119

Murphy L J, Molnar P, Lu X, Huang H 1995 Expression of human insulin-like growth

factor binding protein-3 in transgenic mice. Journal of Molecular Endocrinology 15. 193-303.

Nagy A, Rossant J, Nagy R, Newerly WA, and Roder JC Derivation of completely cell culture-derived mice from early-passage embryonic stem cell 1993 Proc. Natl. Acad. Sci. USA 90:8424-8428

Nivot S, Benelli C, Clot J, Saucet C, Adan L, Souberbielle J, zucker J, Rappaport R, brauner R 1994 Non-parallel changes of growth hormone (GH) and insulin-like growth factor-I, insulin-like growth factor binding protein-3 and GH binding protein after craniospinal irradiation and chemotherapy. J Clin Endocrinol Metab 78:597-601

Nonoshita LD, Wathen NC, Dsupin BA, Chard T, Giudice LC 1994 Insulin-like growth factors (IGFs), IGF-binding proteins (IGFBPs), and proteolyzed IGFBP-3 in embryonic cavities in early human pregnancy:their potential relevance to maternal-embryonic and fetal interaction. J Clin Endocrinol metab 59:899-907

Oh Y, Muller HL, Lamson G, Rosenfeld RG 1993 Insulin-like growth factor (IGF)-independent action of IGF-binding protein-3 in Hs578T human breast cancer cells, J Bio Chem 268:14964-14971.

Oh YM, Nagalla SR,Yamanaka, Kim HS, Wilson E, Rosenfeld RG 1996 Synthesis and

characterization of insulin-like growth factor-binding protein (IGFBP-7)-recombinant human mac25 protein specifically binds IGF-I and II. J Biol Chem 271:30322-30325.

Okajima T, Iwashita M, Takeda Y, Sakamoto S, Tanabe T, Yasuda T, Rosenfeld RG 1993 Inhibitory effects of insulin-like growth factor (IGF)-binding protein-1 and -3 on IGF-activated glucose consumption in mouse BALB/c3T3 fibroblasts. J Endocrinol 136:457-470.

Paszty C, Mohandas N, Stevens ME, Loring JF, Liebhaber SA, Brion CM, Rubin EM 1995 Lethal α-thalassaemia created by gene targeting in mice and its genetic rescue. Nature Genetics 11:33-39.

Potter H, Weir L, Leder P 1984 Enhancer-dependent expression of human kappa immunoglobulin genes introduction into mouse pre-B lymphocytes by electroporation. Proc Natl Acad Sci USA 81 (22): 7161-7165

Pucilowska J, Davenport ML, Kabir I, Clemmons DR, Thissen JP, Butler T, Underwood LE 1993 The effect of dietary protein supplementation on insulin-like growth factor and IGF binding proteins in children with shigellosis. J Clin Endocrinol Metab 77:1516-1521

Rasmussen MH, Frystyk J, Anderson T, Breum L, Christiansen JS, Hilsted J 1994 The impact of obesity, fat distribution, and energy restriction on insulin-like growth factor-I

(IGF-I). IGF binding protein-3, insulin, and growth hormone. Metabolism 43:315-319

Rasmussen MH, Juul A, Kjems LL, Skakkebaek NE, Hilsted J 1995 Lack of stimulation of 24-hour growth hormone release by hypocaloric diet in obesity. J Clin Endocrinol Metab 80:796-801

Rechler MN 1993 insulin-like growth factor binding proteins.Vitam Res 47:1-114.

Ricciarelli E, Hernandez ER, Tedeschi C, Botero LF, Kokia E, Rohan RM, Rosenfeld RG, Albiston AL, Herington AC, Adaski EY 1992 Rat ovarian insulin-like growth factor binding protein-3: a growth hormone-dependent theca-interstitial cell-derived antigonadotropin. Endocrinology 130: 3092-3094.

Rinderknecht E, Humbel RE.1978 The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. J Biol Chem. 253:2769-1776.

Savage MO, Blum WF, Ranke MB, Postel-Vinay MC, Cotterill AM, Hall K, Chatelain PG, Preece MA, Rosenfeld RG 1993 Clinical features and endocrine status in patients with growth hormone insentivity (Laron syndrome). J Clin Endocrinol Metab 77:1465-1471.

Schller AGP, Groffen C, Neck JWV, Zwarthoff EC, Drop SLS 1994 cDNA cloning and

mRNA expression of the six mouse insulin-like growth factor binding proteins. Molecular and Cellular Endocrinology 104:57-66

Schmid C, Rutishauer J, Schlaofer I, Froesch ER, Zapf J 1991 intact but not truncated insulin-like growth factor binding protein-3 (IGFBP-3) blocks IGF-induced stimulation of osteoblasts:control of IGF signalling to bone cell by IGFBP-3-specific proteolysis? Biochem Biophys Res Commun 179:579-585.

Schwander J, Mary JL 1993 The RIA for IGFBP-2 in man-a meager catch? The RIA for IGFBP-2 in man-a meager catch? Growth Regul 3:104-108

Sedivy MJ and Joyner JA 1992 GeneTargeting W.H. Freeman and company, New York.

Shimasaki S, Ling N 1991 Identification and molecular characterization of insulin-like growth factor binding proteins (IGFBP-1, -2, -3, -4, -5 and -6). Prog Growth Factor Res 3:243-266

Shimasaki S, Koba A, Mercado M, Shimonaka M, Ling N 1989 Complementary DNA structure of the high molecular weight rat insulin-like growth factor binding protein (IGF-BP) and tissue distribution of its mRNA. Biochem Biophys Res Commun 165:907-912

Shimasaki S, Shimonaka M, Ui M, Inouye S, Shibata F, and Ling N 1990 Structural

characterization of a follicle-stimulating hormone action inhibitorin porcine ovarian follicular fluid. Its identification as the insulin-like growth factor binding protein. J Biol Chem. 265:2198-2202

Shimasaki S, Shimonaka M, Zhang HP, Ling N 1991 Identification of five different insulin-like growth factor binding protein (IGFBPs) from adult rat serum and molecular cloning of a novel IGFBP-5 in rat and human J Bio Chem 266: 10646-10653

Shimonaka M, Schroeder R, Shimasaki S, Ling N 1989 Identification of a novel binding protein for insulin-like growth factor in adult rat serum. Biochem biophys Res Commun 165:189-195

Smith EP, Dickson BA, Chernausek SD 1990 Insulin-like growth factor binding protein-3 secretion from cultured rat sertoli cells:dual regulation by folicle stimulation hormone and insulin-like growth factor-I. Endocrinology 127:2744-2751.

Smith WJ, Underwood LE, Clemmon DR 1995 Effects of caloric or protein restriction on insulin-like factor-I and IGF-binding proteins in children and adults. J Clin Endocrinol Metab 80:443-449

Smithies O, Gregg R, Bogg S, Koralewski M, Kucherlapati R 1985 Insertion of DNA sequences into the human chromosomal B-globin locus by homologous recombination.

Nature 317:230-234.

Sommer A, Maack CA, Spratt SK, Mascarenhas D, Tressel TJ, Rhodes ET, Lee R, Roumas M, Tatsuno GP, Flynn JA, Gerber N, Taylor J, Cudny H, Nanney L, Hunt TK, Spencer EM 1991 Molecular genetics and actions of recombinant insulin like growth factor binding protein-3. In: Spencer EM (eds) Modern Conceps of insulin-like growth factors. Elsevier, New York, pp 715-728.

Spratt SK, Tassuno GP, Yamanaka MK, Ark BC, Detmer J, Mascarenhas D, Flynn J, Talkington-Verser C, and Spencer E M 1990 Cloning and expression of human insulin-like growth factor binding protein-3. Growth Factors 3: 63-72.

Spratt, SK, Tatsuno GP, and Sommer A 1991 Cloning and characterization of bovine insulin-like growth factor binding protein-3 (bIGFBP-3) .Biochem.Biophys Res Commun. 177, 1025-1032.

Stratagene protocol for Lambda Dash II Library. Instruction Manual, July 12, 1993.

Struhl K 1985 A rapid method for creating recombinant DNA molecules. Biotechniques 3:452-453.

Taaffe DR, Pruitt L, Reim J, Hintz RL, Butterfield GE, Hoffman AR, Marcus R 1994

Effect of recombinant growth hormone on the muscle strength response to resistance exercise in elderly men. J Clin Endocrinol Matab 79:1361-1366

Thomas K and Capecchi M 1987 Site-directed mutagenesis by gene targeting in mouse-embryo-derived stem cells. Cell 51:503-512

Villaudy J, Delbe J, Blat C, Desaulty G, Golde A, Harel L 1991 An IGF binding protein is an inhibitor of IGF stimulation. J Cell Physiol 149:492-496.

Villafuerte BC, Zhang WN, Phillips LS 1996 Insulin and insulin-like growth factor regulate hepatic insulin-like growth factor binding protein-3 by different mechanism. Molecular Endocrinology 10:622-630

WaltonPE, BasterRC, burleigh BD, EthertonTD. Purification of the serum acid-stable insulin-like growth factor binding protein from the pig (sus scrafa). Comp Biochem Physiol.1989; 92B:561-567.

Walton PE, Grant P, Owens JA, Wallace JC, Ballard FJ. Isolation and characterization of bovine plasma IGF binding proteins1,2 and a 24 kDa species. Program of 72nd Annual Meeting of the Endocrine Society 1990; 69 (Abstr. 180).

Walton PE, Gopinath R, Etherton TD 1989 Porcine insulin-like growth factor (IGF)

binding protein blocks IGF-I action on porcine adipose tissue. Pro Soc Exp Bio Med 190:315-319.

Wang HS, Chard T 1992 Chromatographic characterization of insulin-like growth factor binding proteins in human pregnancy serum. J Endocrinol 133: 149-159

Williams RL, Hilton DJ, Pease S, Willison TA, Stewart CL, Gearing DP, Wagner EF, Metcalf D, Nicola NA, Gough NM 1988 Myeloid leukemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature 336:684-687.

Wilson KF, Fielder PJ, Guevara-Aguirre J, Cohen P, Vasconez O, Martinez V, Martinez A, Rosenblood AL, Rosenfeld RG 1995 Long-term effects of insulin-like growth factor(IGF)-I treatment on serum IGFs and IGF binding proteins in adolescent patients with growth hormone receptor deficiency. Clin Endocrinol (Oxf) 42: 399-407.

Wood, W.I., Cachianes, G., Henzel, W. J., Winslow, G. A., Spencer, S. A., Hellmiss, R., Martin, J. L., and Baxter, R. C 1988 Cloning and expression of the growth hormone-dependent insulin-like growth factor binding protein Mol. Endocrinol. 2, 1176-1185

Zapf J, kiefer M, Merryweather J, Masiarz F, Bauer D, Born W, Fischer JA, Froesch ER. Isolation from adult human serum of four insulin-like growth factor (IGF) binding proteins and molecular cloning of one them that is increased by IGFI administration and

in extrapancreatic tumor hypoglycermia.J Bio Chem. 1990; 265:14892-14898.

Zapf J, Hauri C, Wald vogel M, Futo E, Hasler H, Bink K, Guler HP, Schmid C, Froesch 1989 Recombinant human insulin-like growth factor I induces its own specific carrier protein in hypophysectomized and diabetic rats. Proc Natl Acad Sci USA 86:3818-3117

IMAGE EVALUATION
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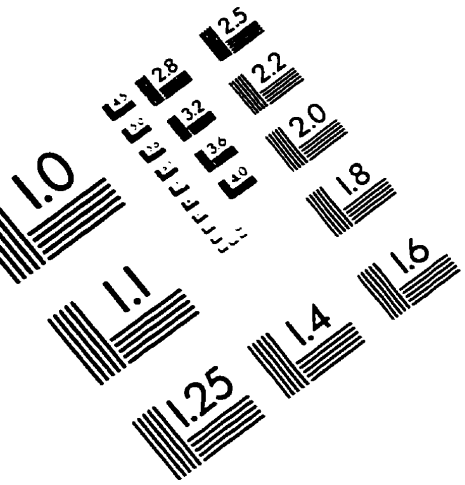

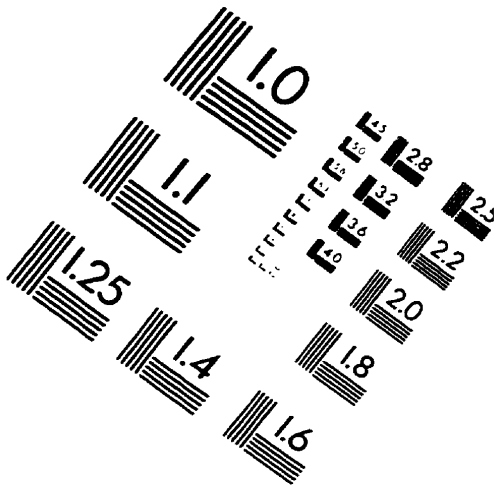

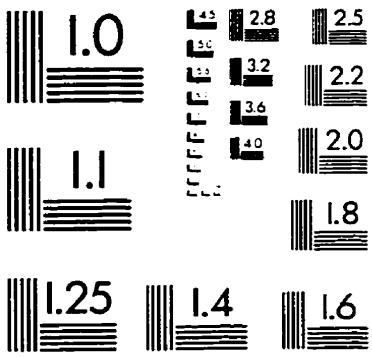

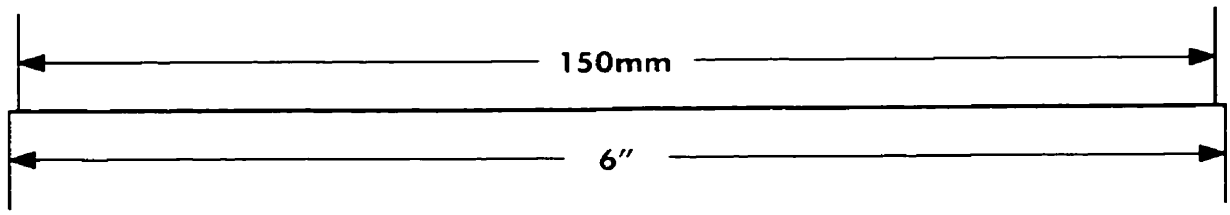

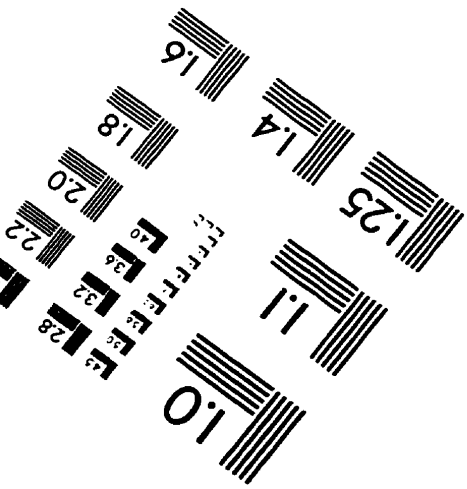

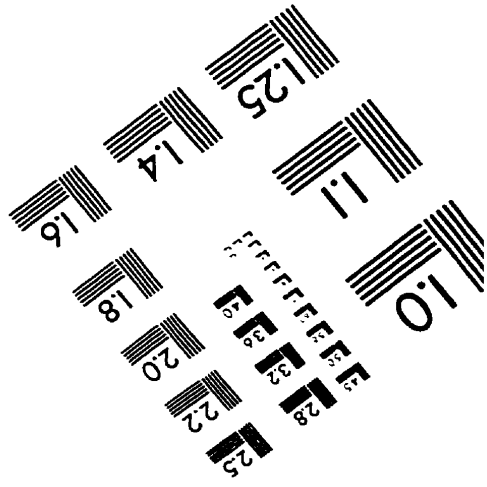

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