Growth Regulation in IGF-1 Receptor Transgenic Mice

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ABSTRACT

To study the signaling effect of insulin-like growth factor- I (IGF-1), transgenic mice containing IGF-1 Receptor (IGF-1R) cDNA fused to metallothionein promoter were produced by DNA microinjection into the pronucleus of mouse zygote. Three founders were produced with transgenic mice containing IGF-1R gene. Transgenic mice lines contained approximately 4～20 copies of transgenes per cell and transmission of this gene into the progeny with Mendelian manner were determined. The founder mice were mated with normal mice to produce F_1 mice and then F_2 mice. Transmission rates of IGF-1R transgene in the progeny mice were 25～60% in F_1 generation and 40～50% in F_2 generation. The mRNA expression of IGF-1R transgene in liver was analyzed using RT-PCR for IGF-1R gene in liver. When body weights of transgenic pups were measured during 4, 10 and 14 weeks after birth, IGF-1R transgenic mice grew faster than non transgenic littermates. This study indicated that growth regulation by IGF-1 signaling through IGF-1R can be elucidated using IGF-1R transgenic mice.

(Key words : Transgenic mice; IGF-1R; RT-PCR; Transmission; Growth rate)

INTRODUCTION

Insulin-like growth factor-I (IGF-1) is essential for cell growth, differentiation and postnatal development and a low molecular weight peptide that mediates the cell proliferating actions of growth hormone (GH). Evidence indicates that IGF-1 is produced by various cell types and acts in a variety of reproductive processes. IGF-1 acts as a negative feedback mediator on the hypothalamus by the production of somatostatin, which inhibits the release of GH from the anterior pituitary. IGF-1 also acts at the level of the pituitary to inhibit expression of the GH gene in response to GHRH. GH induces the synthesis of IGF-1 as well as IGF-1-binding proteins in the liver and other tissues, causing secretion of both IGF-1 and binding proteins into the bloodstream. Consequently, IGF-1 (and other somatomedins) forms complexes with binding proteins in the circulation (Waterfield *et al.*, 1989; McDonald *et al.*, 1989; LeRoith *et al.*, 1995).

The IGFs are essential stimulators of fetal and postnatal growth (Liu *et al*, 1993; 1998; Powell-Braxton *et al.*, 1993). Their signals are mediated predominantly by the Insulin-like growth factor-I receptor (IGF-1R). IGF-1 is a pleiotropic hormone synthesized in a wide variety of cell types (Monica *et al.*, 1999). As its name suggests, it is highly homologous to insulin and has much similar growth promoting effects. IGF-1 is believed to be the major anabolic mediator of growth hormone action (Warburton *et al.*, 1995). During organogenesis, the actions of IGF-1 seem to be local (autocrine/paracrine). IGF-1 is a more potent stimulator of human postnatal growth, and the IGF-1R mediates most IGF actions in humans. The fact that lack of IGF-1 or IGF-1R function is linked to the human growth disorders suggests that mutations in IGF or IGF-1R genes suggests that such defects may cause lethal abnormal development in human (D'Ercole, 1996).

To evaluate the effect of amplification of growth-related receptor signaling through overexpression of receptors on growth regulation in animals, transgenic mice lines were produced by DNA microinjection using metallothionein promoter legated to IGF-1 receptor (IGF-1R) gene in this experiment, and gene expression, body weight, growth pattern and other phenotypes were examined.

MATERIALS AND METHODS

Vector Construction

The pMT-LCR (locus control region) expression vector 2999 (pMT5′3′) contained the mMT-1 promoter and 650 bp of hGH 3′untranslated region and poly A signal. pMT5′3′ has mouse MT locus with 10 kb of 5′ hypersensitive (HS)

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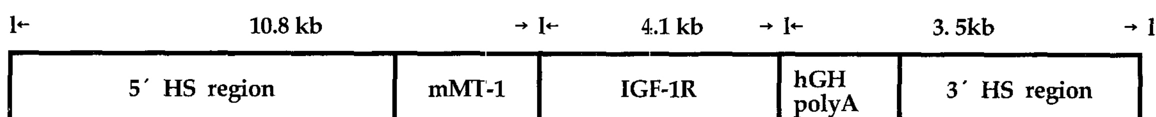

Fig. 1. Construction of MT-IGF-1R gene.

site and 7 kb 3′ hypersensitive site The unique Nru I site of pMT5′3′ was used to insert cDNA of IGF-1R. Transgenes were linearized with Ksp I for microinjection into embryos (Palmiter *et al.*, 1993).

Breeding of Transgenic Mice

The FVB/n female mice over 6 weeks were used in this study. Transgenic mice with IGF-1R genes were generated by the microinjection of cloned DNA into fertilized eggs, followed by embryo transfer into pseudopregnant foster mothers. Transgenic mice were outbred with non-transgenic mice to produce F_1 and F_2 progenies. The mice were maintained on the 14 hr light, 10 hr dark lighting cycle.

DNA Preparation

The transgenic vector sequence was digested with Ksp I to linearize for microinjection. These DNA fragments were diluted with $T_{10}E_{0.1}$ buffer then dialyzed in $T_{10}E_{0.1}$ (10 mM Tris-HCl/0.1 mM EDTA) for 48 hr on ice. The DNA was precipitated with 2 volume of 100 % ethanol and 0.1 volumes of 3 M sodium acetate then centrifuged to obtain the DNA pellet. Then the pellet was diluted with $T_{10}E_{0.1}$ buffer to final concentration of 10~20 ng/μl.

Microinjection and Embryo Transfer

Microinjection was performed under a DIC optic on an inverted microscope (Nicon) fitted with micromanipulator (Nicon, Japan). Approximately 1~2 pl (10~20 ng/μl) DNA was injected into the male pronuclei of the one cell zygote. One-cell embryos for microinjection were collected from the FVB stain female mice. Superovulation was induced by injection of 5 IU PMSG and 5 IU HCG 48 hr apart. Embryos were collected at 20 to 24 hrs post hCG treatment by flushing the oviducts with Dulbeccos PBS. M16 medium and M2 medium as well as CZB medium were used for microinjection. Recipient mice were synchronized by injecting 5 IU hCG and transferred into the oviduct of psedopregnant recipients.

DNA Isolation

DNA was isolated from whole newborn mice tail. Tissue was incubated for overnight at 55℃ in Proteinase K / DNA extraction buffer (50 mM Tris/ 100 mM EDTA/ 10% SDS). The homogenate was extracted twice in PCI (phenol: chloroform: isoamyl alcohol = 25: 24: 1) and once CI (chloroform: isoamyl alcohol = 24: 1). DNA precipitation was carried out with a 2 volume excess of 100 % ethanol and 0.1 volume of 3 M sodium acetate and was air-dried about 5 min and resuspended in $T_{10}E_1$ buffer (10 mM Tris-HCl/ 1 mM EDTA).

Detection of Transgene with PCR Amplification

PCR amplification was performed with the Single Block TM system (ERICOMP Corp, USA). PCR was carried out in 20 μl reaction volume containing 100 ng/μl of DNA solution, 2 mM of the dNTP, 10 pM of the primer (sense and antisense primer), and the 1 unit of Taq polymerase (Promega Corp.). The primer pair used for detection of transgene was forward; 5'-ATCAAAGAGGAGATGGA-GC-3'(for IGF-1R) and reverse; 5'-TTATTAGGACAAG-GCTGGT-3'. The PCR condition was 35cycles of 94℃ for 1 min, 57℃ for 1 min and 72℃ for 1 min. Also 5 min at 94 ℃ was allowed for separation of double strands in the beginning and an extra 7 min at 72℃ was allowed for extension at the end of amplification. After PCR, amplified DNA was separated in 2% agarose gel electrophoresis. The expected size of the IGF-1R gene fragment amplified from cDNA should be 420.

Southern Blot Hybridization

The genomic DNA (10 to 20 μg) was digested with EcoR I which releases about 5.6 kb fragment including IGF-1R cDNA and electophoresed 1 % agarose gel for overnight in 1 × TAE buffer with EtBr (0.5 μg/ml). The gel was denatured in denaturation solution (0.2 mM NaCl/0.6 mM NaCl) for 40 min with shaking at RT and then neutralized in neutralization solution (1 mM Tris/0.6 mM NaCl) for 1 hr. The DNA fragments were transferred to Nylon transfer membrane (Schlecher & Schull Corp.) for overnights in 20 × SSC (1 x SSC: 0.15 M NaCl/0.015 M Sodium citrate) by capillary method. The membrane was rinsed with 2 × SSC and baked in 80℃ for 2 hr. The membrane was prehybridized in hybridization buffer at 42℃ for 2 hr and added ^{32}P-labeled probe for overnight at 42℃. Probe (containing IGF-1R or GHR cDNA) was purified with QIAquick nucleotide removal kit, probe was labeled with ^{32}P by random primer methods. The blot were washed 2 × SSC / 0.1% SDS for 30 min at 68℃ twice and exposed the filter to X-ray film 16 to 24 hrs at -70℃ with an intensifying screen.

RNA Isolation and RT-PCR

Adult (4~8 weeks old) male/female mice were sacrifices by CO_2 saturation and various tissues were kept in liquid nitrogen. Total RNA was isolated from these frozen tissues using TRI REAGENT (molecular research

center, INC, USA). RT-PCR reaction was carried out 20 μl reaction volume (containing 100 mM of $MgSO_4$, 4 μl of 5 × buffer, 2.5 mM of dNTP, 7 μl of ddH_2O, 2.5 units of Reverse Transcriptase, 10 pM of Poly A primer) and 3 attomole of RNA sample. The mixture was incubated at 42 ℃ for 40 min and boiled at 100℃ for 3 min. Immediately mixture was kept on ice and added 100 mM of $MgSO_4$, 4 μl of 5 × buffer, 2.5 mM of dNTP, 57 μl of ddH_2O, 2.5 units of Reverse Transcriptase, 10 pM of upstream primer, 10 pM of downstream primer and 2 mM of DNA polymerase (Access RT-PCR system, Promega Corp, USA). The primer pair used to detect transgene was forward primer 5'-ATCAAAGAGGAGATGGAGC-3'(for IGF-1R) and reverse 5'-TTATTAGGACAAGGCTGGT-3'(for poly A) were used for detection transgenes. The PCR condition was 35 cycles of 94℃ for 1 min, 57℃ for 1 min and 72℃ for 1 min. Also extra 5 min at 94℃ was allowed for separation of double strands at the beginning and an extra 7 min at 72℃ was allowed for extension and the end of amplification. After PCR, DNA was separated in 2% agarose gel electrophoresis. The size of the fragment amplified from cDNA should be 420 bp (IGF-IR gene).

Statistical Analysis

Comparison of body weights between normal and transgenic mice was done by Student *t*-test.

RESULTS AND DISCUSSION

Identification of Transgenic Mice

For detection of IGF-1R transgene, PCR and Southern blot method were used in this study. The PCR is a technique for the *in vitro* application of specific DNA sequences by the simultaneous primer extension of complimentary strands of DNA. Southern blotting was used for the identification of transgene and presumption of copy number using plasmid DNA equivalent to genomic copy number such as 70 pg for 10 copy number and 350 pg for 50 copy number (data not shown). Genomic DNA was extracted from mouse-tail. In both PCR and Southern blotting, a plasmid DNA (IGF-IR) was used as a positive control. Fig. 2 showed that PCR screening of transgenic mice with IGF-1 receptor gene by detection size of transgene was 420 bp. As shown in Fig. 3, IGF-IR transgene was detected with 5.6 kb Eco R1 fragment including IGF-1R cDNA in the transgenic founder mice by Southern blot method. The copy number of lane 1, lane 2 and lane 5 was estimated to be 4, 10 and 20 copies, respectively.

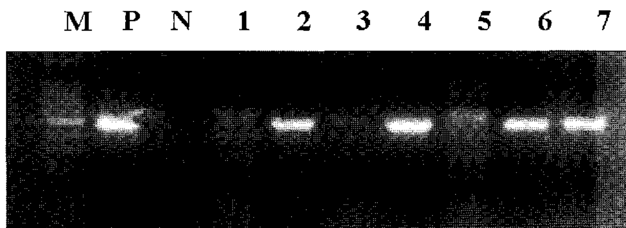

Fig. 2. PCR screening of transgenic mice with IGF-1R gene. M: 100-bp ladder marker, N: negative control, P: positive Control, Lane 2, 4, 6 and 7: transgenic mice with transgene Lane 1, 3, and 5: non-transgenic mice without transgene.

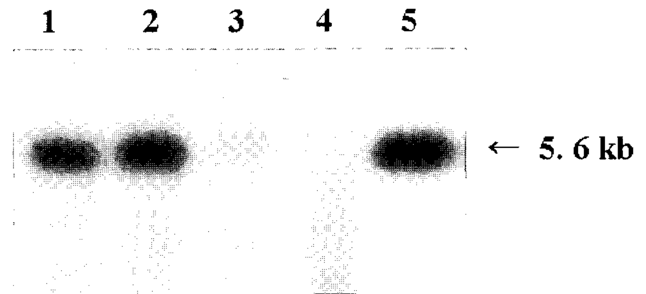

Fig. 3. Identification of transgenic mice with IGF-1R gene by Southern blot analysis (Lane 1, 2 and 5: transgenic mice with transgene, Lane 3 and 4: non-transgenic mice).

Transmission Rate of Transgene

Three founder mice with IGF-1R genes were produced and mated with normal mice. As shown Table 1, transmission rates (no. of transgenics/no. of F_1 or F_2 pups) were 25~66%. Theoretically, founder (F_0) mice with transgene could be 50% by Mendelian fashion unless germline chimera. Transgene was stably transmitted into germline in this experiment conforming that transgene was integrated into mouse genome.

Expression Analysis of mRNA by RT-PCR

RT-PCR was performed to detect expression of IGF-1R genes in transgenic mice. Primers used for this study was specific for IGF-1R transgenics and designed to discriminate endogenous IGF-IR mRNA using reverse primer of Human GH 3′ UTR which was contained in IGF-1R transgene. Fig. 4 showed mRNA expression of transgene using RT-PCR in IGF-IR transgenic mouse liver. The expected size of 420 bp was detected. The band intensity of transgenic mice with IGF-1R (lane 3, 4) was stronger than control mice (lane 1, 2). MT promoter was expressed at the basal level without zinc induction at many organs (including liver, kidney, pancreas, muscle, brain, heart etc.)

Table 1. Transmission rate of transgenic mice with IGR-1R gene in progeny

Lines	Sex of F_0	Transgenics/ F_1 progeny (%)	Transgenics/ F_2 progeny (%)
1	Female	6/9(66.7)	9/23(39.1)
2	Female	3/9(33.3)	11/22(50.0)
3	Male	3/12(25.0)	13/30(43.3)

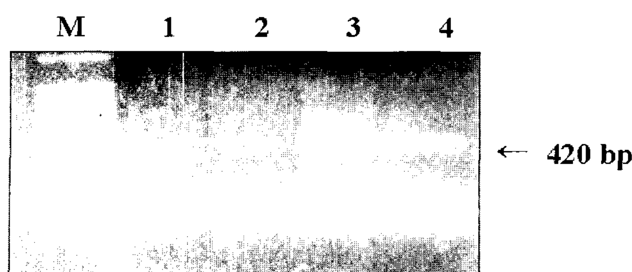

Fig. 4. RT-PCR analysis of IGF-1R mRNA expression in transgenic mice with IGF-1R gene in liver. (M : 100 bp ladder marker, Lane 1, 2 : Control mice, Lane 3, 4 : transgenic female mice with IGF-1R gene).

for all stage of development (Palmiter *et al.*, 1993).

Growth Performance in Transgenic Mice

In Table 2 and 3, body weights of transgenic mice were compared with those of normal mice at 4, 10 and 14 weeks after birth (40 IGF-1R transgenic and 40 non transgenic litter mice for male and female groups). The body weights of transgenic mice were heavier than those of non-transgenic control mice regardless of sex ($P<0.05$). The body weight of transgenic mice was increased 14 to 20% compared with that of control mice The growth rate of transgenic male mice was higher than that of female. For all stages, body weights of transgenic male mice were heavier than transgenic female mice. These results was coincided with IGF-1 transgenic report that although there were no other significant differences between IGF-1 transgenic and control mice, the mean body weights of both male and female IGF-1 transgenic mice were greater than those of normal littermates at 30 days of age (Behringer. *et al.*, 1990).

Table 2. Comparison of body weights between transgenic and normal female mice

Line	4-Week	10-Week	14-Week
Control	14.51±0.6[a]	22.32±0.6[a]	25.51±0.5[a]
IGF-IR	17.34±0.6[b]	24.70±0.4[b]	27.02±0.4[b]

[a] Different superscripts within column denote significant differences ($P<0.05$).
Unit: g±SE.

Table 3. Comparison of body weights between transgenic and normal male mice

Line	4-Week	10-Week	14-Week
Control	18.11±0.5[a]	25.97±0.5[a]	28.53±0.5[a]
IGF-IR	22.13±0.6[b]	28.17±0.6[b]	31.60±0.4[b]

[a] Different superscripts within column denote significant differences ($P<0.05$).
Unit: g±SE.

In conclusion, the results of this study indicate that the application of transgenic technology to improved farm animals basically has a potential to improve farm animals, even if there are some limitations such as lack of knowledge concerning the genetic basis of various factors. To improve the efficiency of transgenic animal production, it needs to identify tissue- and developmentally specific regulatory sequences for transgene constructs, expression vectors and gene targeting system, and establishment of novel transgenic methods. Very little is known about the endogenous expression of the IGFs and the IGF-1R during postnatal stages of animal growth. It needs a further study to understand the molecular mechanism of the IGF-1 system in IGF-1R transgenic mice.

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