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Expression and purification of growth hormone from Microminipig (MMP) and its effects on body fat percentage with injection

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ABSTRACT

Anterior pituitary gland of brain releases the growth hormone. It plays an important role in overall body development of human as well as in animal and it is the candidate gene for gain in body weight. Until now, over expression and purification techniques to obtain soluble growth hormones from mini pig and normal pig are studied. In this study, we have developed the more efficient ways to obtain physiologically active recombinant growth hormone of Microminipig. Microminipig is the smallest pig in the world developed by Fuji Nojo Service, Japan, and the weight of this pig is approximately 7.0 kg at the age of 6 months. Recombinant growth hormone was over expressed as a biologically inactive inclusion body. Inclusion body was solubilized with denaturating agents and refolding was carried out by stepwise dialysis technique. To identify the physiological activity of refolded recombinant GH, we injected it to the Microminipigs (75 µg/kg of body weight). We found that the GH injection induces the more efficient acceleration of muscle growth than that for non-injected Microminipig.

1. INTRODUCTION

Wild boars (*sus scrofa*) were led to development of pigs by domestication. With the use of animal domestication, its selective breeding and adaptation have developed many breeds globally [1]. Pigs are the outstanding model for research in medical because it has similarity with humans in various aspects such as anatomy, physiology etc. Growth hormone 1 (*GH1*) gene is a candidate gene for body weight and weight gain in animal since it plays a fundamental role in growth regulation [2]. Growth hormone (*GH*) was first isolated from human pituitaries in 1956 but the biochemical structure was established only in 1972 [3]. The pig brain has hypothalamus region that contains anterior pituitary gland which secrets porcine growth hormone (pGH). It operates various body metabolisms. The studies related to structure and function of GH shows that, it uses two sites for binding to two receptors [4]. The pGH gene is located on chromosome 12 pl.2~p1.5 [5]. The first 26 amino acid residues of pig GH comprises signal peptide and 190 amino acids as mature protein with two disulphide bridges to form an antiparallel four α-helix bundles [6,7]. *GH* in all mammals extends over 2~3 kb and contains five exons splitted by four introns. The *GH* gene found as a single copy in genome of pig [8]. The 1.7 kb transcribed region of gene consists of five exons [9]. The gene contains four introns of 244, 210, 192 and 275 bp respectively. In higher primates, the *GH* gene has expanded by a series of gene duplications to give a gene cluster. The pleiotropic actions of GH result from its binding to GH receptors (GHRs) which results in the signal transduction of intracellular processes [10, 11] and activates series of metabolic processes which lead to production of IGF-1 by liver tissues [12]. IGF activity is regulated by GH which helps in postnatal somatic growth and stimulation of anabolic processes [13]. The endocrine system of animal plays an important role in animal growth and in the partitioning of nutrients between muscle and adipose tissue [14, 15]. Previous reports demonstrated that the administration of pST to growing pigs markedly increases growth rate, feed efficiency, and muscle growth while dramatically reducing fat accretion [16-19]. Machlin (1972) studied that daily injection of pGH to swine from 47 to 95 kg significantly improved weight gain and feed efficiency [20]. Regulation of growth hormone plays important role in linear growth and its co-ordinated physiological metabolisms in animals. Ethernon and Walton studied that the GH is unique in that it is a catabolic hormone in adipose tissue and an anabolic hormone with respect to protein deposition in muscle [21]. In the meat industry, lean meat and fat content evaluation is important factor. Magnetic resonance imaging (MRI)

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helps to visualize anatomical and pathological changes *in vivo*. The various tissues such as fat and muscle can be identify with the use of magnetic resonant protons in MRI scanning. MRI studies of Mitchell and his coworkers indicated a linear increase in muscle volume, a curvilinear increase in fat volume, and concave pattern for the fat: muscle ratio [22]. In this experiment, we have used the Microminipig (MMP), developed by Fuji Nojo Service, Fujinomiya, Shizuoka Prefecture Kitayama, Japan. The mother of MMPs, "Catherin" is born by crossing of Pot-bellied pig with minipig of another type. MMPs weigh less than 10 kg at its maturity which is suitable for life science research. Microminipigs are convenient from economical point of view for research [23]. In order to study the effect of exogenous treatment of GH in MMP we have over expressed the GH Protein in *E. coli* and refolded by using stepwise dilution method and injected into the body of MMP i.m.

2 MATERIALS AND METHODS

2.1 Reagents

pUC19, pET-21b (+) plasmid (Novagen, Japan) and *E. coli* BL21(DE3) pLysS strain (Novagen, Japan) were used as cloning vector and host strain for protein expression, respectively. All chemicals were of analytical grade or above, and all buffers and chemicals were dissolved in ultrapure water (MilliQ, Millipore).

2.2 Animal sample used and genomic DNA extraction

The present study was conducted at Faculty of Agriculture, Ehime University, Japan. About 30 hairs were picked from the body of MMP and total genomic DNA was extracted by using Qiagen DNeasy kit as per the instructions given. The Quality of genomic DNA was checked by electrophoresis on 1 % DNA agarose gel.

2.3 Polymerase chain reaction (PCR)

Primers were designed for whole *GH* gene fragment and also for amplification of exons based on the coding sequences. The restriction sites were inserted into the each primer for digestion and ligation of exons (Table 1).

The whole *GH* was amplified by using genomic DNA as template. Then each coding part of exon was also amplified by using whole *GH* as a template and further purified for restriction digestion. The reaction mixture contained 7.5 µM of each primer, 1 µl of the template (50 ng/ µl), 2 mM dNTPs, 10× PCR buffer, 25 mM $MgSO_4$ and 1 unit of the KOD-Plus Neo enzyme (Toyobo, Japan). The final volume adjusted to 25 µl with sterile distilled water. The reactions were completed in the GeneAmp® PCR System 9700 and the programme was set as pre-denaturation at 94 °C for 2 min, and 40 cycles with denaturation at 98 °C 10 sec, and extension at 68 °C for 1 min.

2.4 Cloning

The amplified fragments of exons (2 to 5) were digested with Eco RI, Bgl II (Fermentas) and BspH1 (New England Biolabs) respectively and ligated by using ligation High ver 2.0 mixture (Toyobo, Japan) and incubated at 16 °C for 2 hrs. T4 PNK treatment was carried out to catalyze the 5' phosphatase activity. SmaI digested pUC 19 was used and T4 PNK treated product was ligated into it and used to transform competent *E.coli* (strain JM 109) cells. 200 µl of the cells were placed on Luria-Bertani (LB) agar plates containing 50 mg/ml ampicillin allowed to grow overnight at 37 °C. The white transformant colonies were analyzed by colony PCR using Quick Taq™ HS Dye Mix by Toyobo, Japan to check the insert. Restriction digestion was carried out followed by electrophoresis to confirm the correct transformant. Finally, the cloned fragment was cultured and plasmid was extracted by using High Pure Plasmid Isolation Kit (Roche, USA) and sequence of the inserted DNA was checked from both ends using the BigDye® Terminator v3.1 Cycle Sequencing Kit. Sequence data was analysed using the Applied Biosystems sequence scanner v3.1 program. The confirmed sequenced DNA was digested with Xho I and Nde I restriction enzyme and cloned into Xho I and Nde I site of pET21b (+) using ligation High ver 2.0 mixture at 16 °C for 1 hr incubation and transformed into BL21 (DE3) pLysS competent cells to over express the protein.

2.5 Protein expression in *E. coli*

BL21 (DE3) pLysS cells harboring pET21b (+) vector were streaked on LB agar plates containing 50 µg/ml ampicillin and allowed to grow on over night at 37 °C. A single colony from transformed LB agar plate was inoculated into 2 ml of the LB medium (1 % tryptone, 0.5 % yeast extract, and 0.5 % NaCl) containing 100 µg/ml ampicillin grown in shaking incubator at 37°C with 200 rpm. Then culture was inoculated into freshly autoclaved 100 ml LB medium and added 100 µg/ml ampicillin and incubated at 37 °C with horizontal shaking overnight at 200 rpm. Next day, the grown culture was again inoculated into freshly autoclaved 400 ml LB medium (1:5 dilution) containing 50 µg/ml ampicillin and incubated at 37 °C till the OD_{660} reaches 0.4-0.6 in 1 L baffled flask. Samples were collected as before induction (B.I). The expression of recombinant protein was induced by final concentration of 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated for another 3 h at 37 °C. Samples were collected [as an after induction (A.I.)] to check the protein expression by SDS-PAGE. The cells were harvested by centrifugation at 6,000 rpm for 20 min at 4 °C. The cell pellets was washed with 0.15 M NaCl and centrifuged at 4000 rpm for 20 min at 4 °C and pellet were stored at -80 °C overnight. Protein Expression was checked by comparing the samples before induction of pGH protein and after induction of pGH protein by SDS-PAGE.

2.6 Isolation of Inclusion bodies (IBs)

The frozen bacterial pellets were thawed on ice and mixed in 4 ml/gm of pellet in lysis buffer [1M Tris-HCl (pH 8.0), 3 M KCl and 0.5 M EDTA], 7 mM β-mercaptoethanol and 10 mM Amidinophenylmethylsulfonyl fluoride (APMSF). The Cells were disrupted by sonication at output 4, Duty 60 (for 4 min x 6 times) on ice by using TOMY Ultrasonic Disruptor UD-201 and

Fig. 1: Pig *GH* gene structure and primer designed for cloning of MMP *GH*. Yellow highlighted color sequence shows full *GH* forward and reverse primer sequence. Sequence with light blue box is signal peptide sequence. Sequence in capital indicates the coding sequence for mature protein formation. One to Five exons marked by 5 different colors (Brown, Red, Green, Pink and Blue Respectively). Arrows with each color indicates the sequence of forward and reverse primers used for amplification of all exons and at the end of primers restriction site is inserted so that it could maintain the basic amino acid sequence of pig *GH*. Stop codon (TAG) is indicated by red color box. TTCCCA sequence where amino acid was changed to methionine and Glycine respectively to start the mature protein formation.

centrifuged at 15,000 rpm for 20 min at 4 °C. This protein was expressed as IBs. The supernatant and IBs were collected for SDS-PAGE. Remaining supernatant was discarded and the IB pellets were again resuspended in lysis buffer and centrifuged at 15,000 rpm for 20 min at 4 °C. These IBs were precipitated with the 80 % and 90 % Acetone by centrifugation at 15,000 rpm for 20 min at 4 °C. The pellets were dried to form the powder. Concentration of dried acetone powder of GH was measured on Beckman DU® 640 spectrophotometer by using Bradford Assay (24).

2.7 Solubilization of protein from IBs

The collected powder were resuspended in 6 M Guanidine Hydrochloride (GnHCl) containing 1 mM DTT for overnight at room temperature and then centrifuged at 15,000 rpm for 20 min at 4 °C. The insoluble material was discarded. The solubilized protein was dialyzed using dialysis tube (MWCO 8000; nacalai tesque) in buffer containing 8 M urea, 5 mM K_2HPO_4, 45 mM KH_2PO_4 and 1 mM DTT, under constant stirring on magnetic stirrers at 4 °C. This buffer was changed every 12 hrs buffer for 2 days. Concentration of solubilized product was determined by Bradford assay.

2.8 SDS-PAGE

SDS-PAGE was carried out according to method described by Laemmli (25). SDS-PAGE was carried out on 15 % (w/v) acrylamide gel in a discontinuous Laemmli system on the ATTO, Japan at constant current 25 for about 1.5 hr. All the protein samples were prepared with 2X SDS loading buffer and incubated at 95 °C for 5 min. LMW protein marker was loaded for reference. Protein bands were visualized by staining with Quick-CBB (provided by Wako Pure Chemical Industries, Ltd Japan).

2.9 Refolding and dialysis of MMP GH

The dialysate was subjected to a step wise dialysis protocol to refold the GH protein. In this protocol, the concentration of 8 M urea was decreased stepwise to 0 M in presence of 10 mM NH_4HCO_3 containing 0.5 mM DTT because of disulphide protein. Complete removal of strong denaturating

agents such as 6 M GnHCl and 8 M Urea ensures correct refolding of GH protein. Refolded protein was dialyzed against 10 mM NH_4HCO_3 containing 0.5 mM DTT twice at 12 hrs interval. After dialysis concentration of refolded GH protein was measured by Bradford method. The protein was stored in 4 °C**.** 600 µg of refolded protein was freeze dried in eppendorf tube by using freeze dry system supplied by Labconco Company.

2.10 Injection of refolded GH Protein

One pair of each Male and female MMPs having age of three to four months were received from Fuji Nojo Service group. They were adapted under laboratory environment for two months and used for injection of GH experiments. The MMPs were injected with GH by intramuscularly at the concentration of 75 µg/kg of body weight per day. The injection volume for control animal was similar to that used for GH treated pigs with buffer only. The injection was given to each pair of MMP in such way male (control and positive) and female (control and positive) 6 times per week at 7.00 PM daily for continuously 5 months. Specially designed food (Fuji Micra Inc) was supplied to MMP 25 g/kg of body weight/day in evening. Body weight was measured daily before injection.

3 RESULTS AND DISCUSSION

3.1 Amplification of *GH*, Cloning and sequencing analysis of MMP *GH*

The PCR amplification of whole *GH* gene resulted in a 1.79 Kb fragment by using total genomic DNA as a template (Fig 2 a). The amplified whole *GH* product was used for amplification of each coding exon separately (from coding exon 2 to 5 and coding exon 1 includes in signal peptide sequence). All coding exons were amplified successfully with the product size 93 bp, 117 bp, 162 bp, 201 bp respectively (Fig 2 b). The DNA sequence of *GH* consists of 651 bp open reading frame which encodes 216 amino acids among which 26 amino acids encodes for signal peptide.

The PCR amplified product was digested by using restriction enzymes and ligated (Table 2). The ligated product of 573 bp was cloned at SmaI site in pUC19 and confirmed by sequencing with the use of M13 primers which is present at the opposite ends of multiple cloning sites (MCS) of vector. The obtained full length DNA sequence and its encoding amino acids are shown in (Fig. 3). The sequenced product was cloned into pET21b (+) with Xho I and Nde I site and again both strands were confirmed by sequencing. Calculated molecular weight of mature 190 coding amino acids is 21.6 kDa and predicted theoretical pI is 6.83 [26].

3.2 Expression, solubilization and Purification of MMP GH protein

After IPTG induction, protein was expressed as a major protein at 21.6 kDa in the total protein of *E. coli*, which is shown in (Fig. 4 a) The size of expressed protein was in good agreement with the theoretical molecular weight from the amino acid

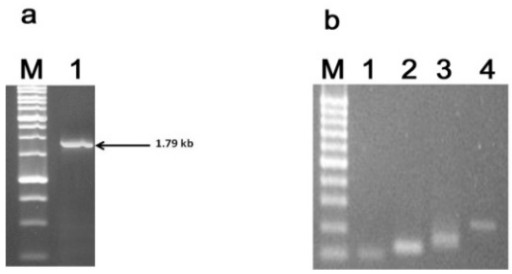

Fig. 2: Agarose gel electrophoresis a) M represents 1 kb marker and 1 represents 1.79 kb fragment of full GH gene. b) M represents 100 bp marker and 1, 2, 3, 4 represents amplification of coding exon 2,3,4,5 with the size of 93 bp, 117 bp, 162 bp and 201 bp respectively.

```
  1 atgggagccatgcccttgtccagcctatttgccaacgccgtgctc
    M  G  A  M  P  L  S  S  L  F  A  N  A  V  L
 46 cgggcccagcacctgcaccaactggctgccgacacctacaaggaa
    R  A  Q  H  L  H  Q  L  A  A  D  T  Y  K  E
 91 ttcgagcgcgcctacatcccggagggacagaggtactccatccag
    F  E  R  A  Y  I  P  E  G  Q  R  Y  S  I  Q
136 aacgcccaggctgccttctgcttctcggagaccatcccggccccc
    N  A  Q  A  A  F  C  F  S  E  T  I  P  A  P
181 acgggcaaggacgaggcccagcagagatctgacgtggagctgctg
    T  G  K  D  E  A  Q  Q  R  S  D  V  E  L  L
226 cgcttctcgctgctgctcatccagtcgtggctcgggcccgtgcag
    R  F  S  L  L  L  I  Q  S  W  L  G  P  V  Q
271 ttcctcagcagggtcttcaccaacagcctggtgtttggcacctca
    F  L  S  R  V  F  T  N  S  L  V  F  G  T  S
316 gaccgcgtctacgagaagctgaaggacctggaggagggcatccag
    D  R  V  Y  E  K  L  K  D  L  E  E  G  I  Q
361 gccctcatgagggagctggaggatggcagcccccgggcaggacag
    A  L  M  R  E  L  E  D  G  S  P  R  A  G  Q
406 atcctcaagcaaacctacgacaaatttgacacaaacttgcgcagt
    I  L  K  Q  T  Y  D  K  F  D  T  N  L  R  S
451 gatgacgcgctgcttaagaactacgggctgctctcctgcttcaag
    D  D  A  L  L  K  N  Y  G  L  L  S  C  F  K
496 aaggacctgcacaaggctgagacatacctgcgggtcatgaagtgt
    K  D  L  H  K  A  E  T  Y  L  R  V  M  K  C
541 cgccgcttcgtggagagcagctgtgccttctaa 573
    R  R  F  V  E  S  S  C  A  F  *
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Fig. 3: DNA sequence of recombinant growth hormone of MMP (with mature protein sequence). All nucleotide represents 190 amino acids.

sequence of the GH. The expressed protein was disrupted with high intensity of sound waves which allows extracting the protein from tissue that has broken open. This protein was expressed mainly as IBs. Because of high density, IBs can be prepared based on principle of liquid and solid separation i.e. by centrifugation [27]. Obtained inclusion bodies were checked by SDS-PAGE for its quality (Fig. 4 b). Further Inclusion bodies were precipitated with 80 % and 90 % acetone respectively. Acetone precipitation helps in downstream applications of protein purification. Recovery of acetone powder obtained after precipitation was about 80 - 85 %. *E. coli* containing IBs are solubilized in high concentrations of denaturants, like 6 M GnHCl and or 8 M urea with or without reducing agent [28, 29]. IBs proteins have native-like secondary structure [30, 31] and when it solubilized it retains the native-like secondary structure which result in high recovery of the bioactive protein [32]. In this experiment dried acetone powder (containing 2.5 – 3.0 mg/ml final concentration of protein) was dissolved in strong denaturing agents like 6 M GnHCl comprising 1 mM DTT as a reducing agent. DTT keeps cysteines in reduced form and breaks the S-S bonds. Completely solubilized sample was dialyzed against 8 M urea. No deposits were found during 8 M urea dialysis

process. SDS-PAGE after 8 M dialysis showed that, protein was completely intact (Fig 4 c). 1.5 mg/ml protein of GH is obtained after 8 M urea solubilization.

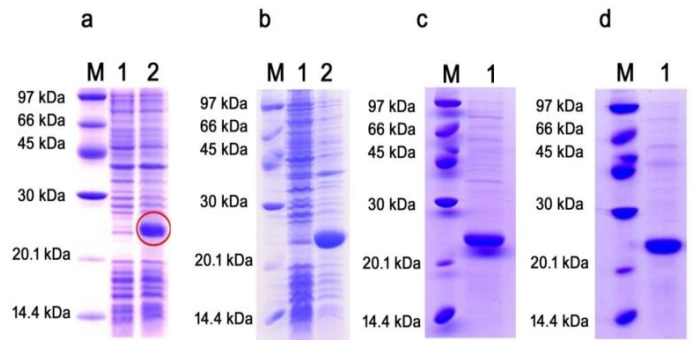

Fig. 4: Expression, purification and refolding of GH protein. Lane M in all the SDS-PAGE represents LMW marker ranging from 14.4 kDa to 97 kDa **a)** Lane 1 shows the protein sample before induction i.e. before addition of IPTG and lane 2 shows sample after induction i.e. after addition of 1 mM IPTG **b)** Lane 1 shows supernatant after cell disruption and lane 2 shows IBs after cell disruption. GH is expressed as IBs **c**) Lane 1 is sample after 8 M Urea solubilization **d)** Lane 1 shows sample after refolding.

3.3 Refolding of GH

Refolding is a process in which the protein conformation is changed to its native form by using strong denaturant [33]. The bond between internal protein is less than the bonds between that of chaotrope and the protein which is solubilized by strong denaturant. The breaking of disulphide bonds under reduced state is important [34]. Removal of 6 M GnHCl and or 8 M urea from solubilized protein, refolds the protein in their native state [35]. Protein was refolded by step wise dialysis process. Refolding was done by reducing the concentration of 8 M urea to 0 M urea in presence of 10 mM NH_4HCO_3 containing 0.5 mM DTT. Refolded sample did not show any turbidity during and after refolding process. The quality of refolded protein was checked by SDS-PAGE (Fig 4 d). Concentration of refolded protein was measured by Bradford method.

3.4 Injection of refolded GH

Our previous short term study showed that, the dose more than 75 µg/kg of body weight per day increases the mobility problem like difficulty in getting up and standing to the MMPs. Exogenous dose of refolded GH 75 µg/kg of body weight per day was injected to MMP six times per week for five months. In case of male, the control and injected MMPs gain 207 and 215 % of starting body weight and female MMPs, the control and injected pigs gain 65.5 and 68.1% of starting body weight respectively. The rates for weight gain in injected MMPs were relatively larger than those for control in both the male and female MMPs. These results suggest that the GH injection accelerate the growth of pigs both in male and female. We also checked the body fat percentage for female pigs with the MRI scan. The results for MRI scan showed that, the body fat percentage was decreased with the GH injection (Data not shown). Chung and his co workers showed that, injection of 22 µg of pGH/kg for 30 d increased rate of growth in rapidly growing, young swine approximately 10 % and improved feed efficiency and also the injection of pGH increased soft tissue mass (i.e muscle + adipose) approximately 6 % [16]. In consideration of above results, we can conclude that, GH injection induces the more efficient acceleration of muscle growth than that of fats in both male and female MMPs. Increase in muscle mass is due to a stimulatory effect of GH on protein synthesis. The pST treated pigs had decreased the raw subcutaneous total fatty acids for 16:0, 18:0, and 18:1(n-9)c and in case of intermuscular fatty acids, the 16:0 and 20:0 decreased most when pigs were treated with pST [36]. Campbell and his co workers found that, injection of pGH with 90 µg/kg of body wt per day with additional dietary protein (17.6 % and 20.7 %) enhances the rate of protein deposition and decreases the fat content of carcass [37].

Table. 1: List of primers used for amplification of whole *gh* gene of MMP and its Exons underlined sequence represents the restriction sites inserted.

Primer	Region	Sequence
GH F	Full *GH*	5'-AGAGGGAGAGAGAAGAGGCC-3'
GH R		5'-TACGATGCAACCTGGTTTTATTAG-3'
F1	Exon 2	5'-CCAGTCATATGGGAGCCATGCCCTTGTCCA-3'
R1		5'-GTCGAATTCCTTGTAGGTGTCGGCAGCC-3'
F2	Exon 3	5'-CAAGGAATTCGAGCGCGCCTACATCCCGG-3'
R2		5'-CTGACAGATCTCTGCTGGGCCTCGTC-3'
F3	Exon 4	5'-GACTGAGATCTGACGTGGAGCTGCTGCGCT-3'
R3		5'-GAGCCTCATGAGGGCCTGGATGCCCTCCTC-3'
F4	Exon 5	5'-CAGTCTCATGAGGGAGCTGGAGGATGGCAGCCC-3'
R4		5'-CGTCCTCGAGTTATTAGAAGGCACAGCTGCTCTCC-3'

Table. 2: Tabular format for conditions of digestion and ligation of all coding exons of *GH* gene.

Exons	Name of RE	Digestion condition	Ligation condition
Exon 2 and 3	EcoRI G▼AATTC CTTAA▼G	37 ℃, 15 min	16 ℃, 30 min
Exon 2 and 3 and Exon 4	BglII A▼GATCT TCTAG↑G	37 ℃, 30 min	16 ℃, 30 min
Exon 2 + 3 + 4 and Exon 5	BspHI T▼CATGA AGTAC↑T	37 ℃, 60 min	16 ℃, 120 min

The body weight of both injected male and female MMPs could not reach to normal pigs and mini pigs. This strongly suggests that the amount of the GH secretion must be one of the reasons for the mini size of MMPs, however, other reason like mutations in the *GH* gene, GH promoter or in Growth Hormone Receptor (GHR) proteins might be playing important role in inducing small size of MMP. Further studies related to *GH* gene structure from MMPs are needed to understand the reason.

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