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CLONING OF BASIC FIBROBLAST GROWTH FACTOR FROM CHINESE SMALL TAIL HAN SHEEP AND ITS EFFECTS ON PROLIFERATION OF MURINE C2C12 MYOBLASTS

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Summary: To further understand characteristics and functions of basic fibroblast growth factor (bFGF, FGF-2) as well as its effects on skeletal muscle, a coding sequence of 18kDa-bFGF gene was cloned from Chinese Small Tail Han sheep, an excellent indigenous animal breed in China. The DNA sequence and deduced protein sequence were analyzed and aligned with the counterparts of other published animals. The results indicated that most hallmarks of the newly cloned bFGF gene sequence were found to be similar to those of published species, which implied further that bFGF genes have been conservative in evolution. The complete coding sequence for the gene was inserted into pET-28a plasmid and expressed predicted fusion protein in E. coli. The product was soluble and possessed of biological activity, it is sure to be a good source for further investigation. In addition, effects of the products on proliferation of murine C2C12 myoblasts were investigated. The findings demonstrated that the ovine recombinant bFGF accelerated in vitro the proliferation of murine C2C12 myoblasts, and MEK/ERK-MAPK signaling pathway participated in the regulative mechanism.

Key words: bFGF; cloning; C2C12; MEK/ERK; Small Tail Han sheep

Introduction

Basic fibroblast growth factor(bFGF, FGF-2) is a member of the FGF family, which comprises twenty-two members that are structurally related polypeptide growth factors found in organisms ranging from nematodes to humans and encoded by twenty-two distinct genes in mouse(1). The protein is a single-chain polypeptide composed of 146 amino acids firstly purified from bovine pituitary. Five forms of the protein, including 18,22,22.5,24 and 34kDa, possessing of identical C-terminal sequences, result from different initiation codon of the same transcript. The 18kDa form is produced by initiation of translation at AUG codon and all the other four forms come from initiation of translation at CUG codons located 5' upstream from the AUG codon. Generally, 22,22.5,24,34 kDa-bFGF are designated as high molecular weight (HMW) forms bFGF (2,3). The 18kDa-bFGF is localized primarily in the cytoplasm whereas the HMW-bFGFs are predominantly localized in the nucleus (4), which imply that they may perform different biology roles through respective signal pathways.

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Basic FGF was initially identified as a mitogen with prominent angiogenic properties, but now was recognized as a pleiotropic effector in different cells and organ systems (5), a key function is the ability to stimulate proliferation of cells derived from mesodermal and neuroectodermal tissues (6), and found in all organs, solid tissues, tumors and cultured cells including muscle cells (7-12). Basic FGF is regarded as a potent stimulator of the proliferation and fusion of myoblasts in vitro and a factor to enhance muscle regeneration in vivo (13,14). Impaired expression of endogenous bFGF gene in rat skeletal muscle caused by ischemia and reperfusion may be a reason in delayed wound healing (15,16). Furthermore, it has been reported that there is a positive relationship between bFGF and the speed and success of muscle regeneration (17). During the skeletal muscle course of development and regeneration, many growth factors exert their biological roles, for example, bFGF and myostatin, the former improves proliferation of C2C12 myoblasts and the latter plays an opposite role. Our previous reports revealed the suppression of bFGF on endogenous expression of myostatin gene in C2C12 myoblasts and the participation of ERK/MEK-MAPK signaling pathway in the regulatory process(18,19).

It has been proved that bFGF is highly conservation among all published species and performs biological roles on development, wound repair and regeneration of skeletal muscle. To understand the evolution of bFGF on a deeper time scale and effects on skeletal muscle, in this paper we report the molecular cloning and characterics of 18kDa-bFGF orthologous genes expressed in Chinese Small Tail Han sheep (CSTHS), a famous indigenous Chinese sheep breed, and expression and purification of the protein overproduced in *E.coli* (BL21). In addition, effects and regulative mechanism of bFGF on proliferation C2C12 myoblasts are also discussed.

Materials and methods

PCR amplifys complete 18kDa-bFGF gene of Small Tail Han sheep

Total RNA of CSTHS was isolated from spleen preserved in author's laboratory with Trizol reagent according to the manufacturer's protocol. For the amplification of 18kDa-bFGF gene, a set of primers was synthesized based on our published CSTHS 18kDa-bFGF cDNA sequence (GenBank accession numbers: DQ091182). 5'-GATATCATGGCCGCCGGGAGCATCA-3' and 5'-GTCGACTCAGCTCTTAGCAGACATTGG-3' were chosen as the sense primer and antisense primer. The PCR products were ligated into pMD18-T vector and the ligated products were used for transforming *E.coli* DH5α competent cells. The recombinant plasmid DNA, pMD18-T/obFGF, was isolated and sequenced, and protein putative structure of bFGF was deduced according to DNA sequences. DNA and protein sequences were compared with the counterparts of other published species. Database searches and sequence alignment were performed at the National Center for Biotechnology Information and with DNAMAN Software.

Construction of expression plasmid

To construct an expression vector, a set of primers 5'-ATGAATTCATGGCCGCCGGGAGCATCA-3' carrying an *EcoRI* site and 5'-GCACTCGAGAGCTCTTAGCAGACAT-3' containing an *XhoI* site were employed to amplify bFGF complete coding seuqence from plasmid pMD18-T/obFGF. The amplification products were digested with *EcoRI* and *XhoI* endodeoxyribonuclease, and then ligated into vector pET-28a that had been previously cut with the same enzymes, the final vector pET-28a/obFGF was resulted. DNA of pET-28a/obFGF was produced by transforming into *E.coli* DH5α and transformed into *E.coli* BL21. The sequence of the insert was confirmed using automated DNA sequencing.

Expression and purification of ovine recombinant bFGF(or-bFGF)

E.coli cells harboring pET-28a/obFGF were grown in 100 ml of LB medium containing kanamycin at 37℃. When the A600 reached 0.6-0.8, the culture was added to 1 L of LB medium containing kanamycin and incubated at 37℃. When the A600 reached 0.6-0.8 again, isopropyl-β-D-thiogalactoside was added to a final concentration of 0.5mM and the culture was continued at 25℃ for 5 h. Cells were harvested by centrifugation at 5000g for 15min at 4℃ and the cell pellet was washed with ice-cold binding buffer

containing 20mM sodium phosphate and 500mM sodium chloride adjusted to pH7.8 with H_3PO_4. Cells were resuspended in 20ml of binding buffer containing 1mM PMSF and 1mg/ml lysozyme and incubated at 4℃ for 30min. The suspension was sonicated and then centrifuged at 12000g for 20min at 4℃. The supernatant was harvested and applied to a Ni-NTA column equilibrated with binding buffer. The column was washed with washing buffer(20mM sodium phosphate, 500mM sodium chloride) adjusted to pH6.0 with H_3PO_4 and supplemented with 20mM imidazole, until the A280 was less than 0.01. Proteins were eluted with elution buffer containing 20mM sodium phosphate, 500mM sodium chloride and 250mM imidazole. Fractions containing most of the A280 material were pooled. The purity of or-bFGF in column fractions was assessed using SDS-PAGE. The purified protein was dialysed in dialysis buffer (5mM Tris-HCl, pH7.6). Protein concentration was determined by bicinchoninic acid assay. The protein was freeze-dried and stored at -80℃.

Immunoblotting analysis

Protein samples were denatured in sample buffer for 5 min in boiling water and loaded onto SDS-PAGE. Electrophoresis was carried out before the protein was transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in TBS containing 0.05% Tween-20. Then, the membranes were incubated with polyclonal antibody against human bFGF for 1hr. Secondary antibody conjugated with horseradish peroxidase was incubated after washing with TBS containing 0.05% Tween-20 for 1 h. The blot was washed four times and colored using DAB kit according to the manufacturer's protocol.

Activity assay of fusion protein

The biological activity of or-bFGF protein was detected by the induction of proliferation of 3T3 fibroblasts. NIH-3T3 fibroblasts diluted to 1×10^5 / ml were grown in DMEM supplemented with 10% fetal bovine serum(FBS). When the cells was about 80% confluent, cells were transferred into a 96-well plate and incubated in DMEM supplemented with 10% FBS for 8 h, with five replicates of each well, then the medium was changed to DMEM supplemented with 0.5% FBS and incubated for 48 h. Cells were incubated in medium (containing different concentrations of either human recombinant bFGF(hr-bFGF, Sigma) or or-bFGF, 10ng/ml heparin, and 0.5%FBS) for 48 h, methylthiazoletetrazolium solution in concentration of 5mg per ml was added into wells. After 6hr, each well was added with 100µl DMSO. The plate was vibrated for 20min on a vibrator. Optical density values were read in enzyme-linked immunosorbent assay instrument, at wavelength 570nm.

Murine C2C12 myoblasts proliferation assay

Murine C2C12 myoblasts were grown to 80% confluence in DMEM with 10% FBS. Cells were harvested, diluted to 1×10^5 /ml, added in quintuplicate to each well of 96-well tissue culture plate and incubated for 24 h. Medium was replaced with DMEM containing 0.5% FBS and incubated for 48 h. The medium was replaced with DMEM containing 0.5% FBS, different concentrations of or-bFGF and 10ng/ml heparin. The following incubation was performed for 48 h and MTT was added into wells. Followed incubation for 6 h, wells were added with 100µl of DMSO and sufficiently vibrated for 20min. Optical density values at wavelength 570nm were measured.

Effects of inhibitors of MAP kinase signaling pathways on proliferation of C2C12 myoblasts induced by bFGF

C2C12, grown to 80% confluence in DMEM with 10% FBS, were plated in quintuplicate into each well of 96-well tissue culture plate at a density of 1×10^5 /ml and incubated for 24 h. Myoblasts were transferred to DMEM with 0.5% FBS and incubated for 48 h. Cells were pretreated for 1h with medium containing 0.5% FBS and inhibitor (10µM SB203580, 50µM PD98059 or 10µM SP600125). Cells were transferred into medium containing 0.5% FBS, 20ng/ml or-bFGF and 10ng/ml heparin. The following incubation was performed for 48 h. MTT assay was employed to detect cell proliferative activity.

Data and statistical analysis

Experimental data were analyzed by ANOVA using the SPSS statistical software. Results were expressed as the means and standard errors. Significance was considered at $p<0.05$ or $p<0.01$.

Results

Molecular characteristics

Nucleotide sequence for the coding region of bFGF cDNA from Small Tail Han sheep is different to the other published sequences, but all known 18kDa-bFGF orthologous gene sequences remain highly conservation during evolution. The similarities of coding nucleotide sequences between Small Tail Han sheep and other published terrestrial animals were found to be 99.8% (cattle, NM174056), 98.9% (sheep, NM001009769), 95.3% (chimpanzee, AY665259), 95.1% (human, NM002006; monkey, XM001099284), 94.2% (dog, XM533298), 90.8% (house mouse, NM008006), 89.5% (Norway rat, NM019305), 87.4% (gray short-tailed opossum, NM001033976), and 84.8% (non-mammalian chicken, NM205433) respectively. The similarities between the sheep and other published amphibian were 78.0% (Japanese firebelly newt, AB064664) and 75.9% (silurana tropica, NM001017333), and the similarities between the sheep and other published aquatic animals were 69.7% (zebrafish, NM212823) and 67.9% (rainbow trout, AY878375).

Deduced amino acid sequence of 18kDa-bFGF from Small Tail Han sheep was aligned with NCBI and DNAMAN software. The results showed that polypeptide sequences among all species were conservative and the similarities of Small Tail Han sheep protein primary structure to that of other published species were 99.4% (cattle), 98.7% (dog, sheep), 98.1% (human, chimpanzee and monkey), 97.4% (Norway rat), 95.5% (house mouse), 94.2%(gray short-tailed opossum), 93.5% (chicken), 86.5% (Japanese firebelly newt), 82.5% (silurana tropica), 74.7% (zebrafish) and 74.2% (rainbow trout), respectively. Nucleotide and amino acid sequences were given in Figure 1.

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1     ATG GCC GCC GGG AGC ATC ACC ACG CTG CCA GCC CTG CCG GAG GAC GGC GGC GGC GGC GCT
1      M   A   A   G   S   I   T   T   L   P   A   L   P   E   D   G   G   G   G   A
61    TTC CCG CCG GGC CAC TTC AAG GAC CCC AAG CGG CTG TAC TGC AAG AAC GGG GGC TTC TTC
21     F   P   P   G   H   F   K   D   P   K   R   L   Y   C   K   N   G   G   F   F
121   CTG CGC ATC CAC CCC GAC GGC CGA GTG GAC GGG GTC CGC GAG AAG AGC GAC CCA CAC ATC
41     L   R   I   H   P   D   G   R   V   D   G   V   R   E   K   S   D   P   H   I
181   AAA CTA CAA CTT CAA GCA GAA GAG AGA GGG GTT GTG TCT ATC AAA GGA GTG TGT GCA AAC
61     K   L   Q   L   Q   A   E   E   R   G   V   V   S   I   K   G   V   C   A   N
241   CGT TAC CTT GCT ATG AAA GAA GAT GGA AGA TTA CTA GCT TCT AAA TGT GTT ACA GAC GAG
81     R   Y   L   A   M   K   E   D   G   R   L   L   A   S   K   C   V   T   D   E
301   TGT TTC TTT TTT GAA CGA TTG GAG TCT AAT AAC TAC AAT ACT TAC CGG TCA AGG AAA TAC
101    C   F   F   F   E   R   L   E   S   N   N   Y   N   T   Y   R   S   R   K   Y
361   TCC AGT TGG TAT GTG GCA CTG AAA CGA ACT GGG CAG TAT AAA CTT GGA CCC AAA ACA GGA
121    S   S   W   Y   V   A   L   K   R   T   G   Q   Y   K   L   G   P   K   T   G
421     CCT GGG CAG AAA GCT ATA CTT TTT CTT CCA ATG TCT GCT AAG AGC TGA
141     P   G   Q   K   A   I   L   F   L   P   M   S   A   K   S   *
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Figure 1: The nucleotide sequence and deduced amino acid sequence of basic fibroblast growth factor from Chinese Small Tail Han sheep

Expression, purification and activity assay of or-bFGF

A 22~kDa predicted protein was induced in the presence of IPTG(Figure 2), the mature recombinant protein comprised full 18kDa-bFGF. The protein was purified by immobilized metal affinity chromatography (Figure 3A) and recognized by antibodies against human recombinant bFGF on western blots(Figure 3B). These results indicated that a predicted fusion protein of bFGF was produced by *E.coli*. containing recombinant plasmid pET-28a/obFGF. In addition, we also determined whether bFGF fused to His-Tag retained biological activity on proliferation of fibroblasts. It was revealed that or-bFGF stimulated the growth of NIH-3T3 cells (Table 1). We also found that or-bFGF stimulated the growth of NIH-3T3 cells to the same extent as human recombinant bFGF (hr-bFGF).Thus, or-bFGF retains its biological activity when fused to His-Tag, providing a good source for further investigation.

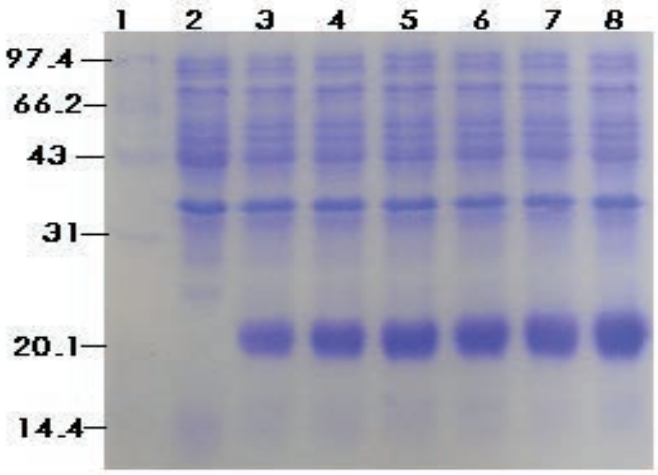

Figure 2: Expression of or-bFGF in *E.coli*. After treating BL21 cells containing pET-28a/bFGF(lane 3~8) or pET-28a(lane 2) with IPTG. Cell lysates were separated on a SDS-PAGE gel

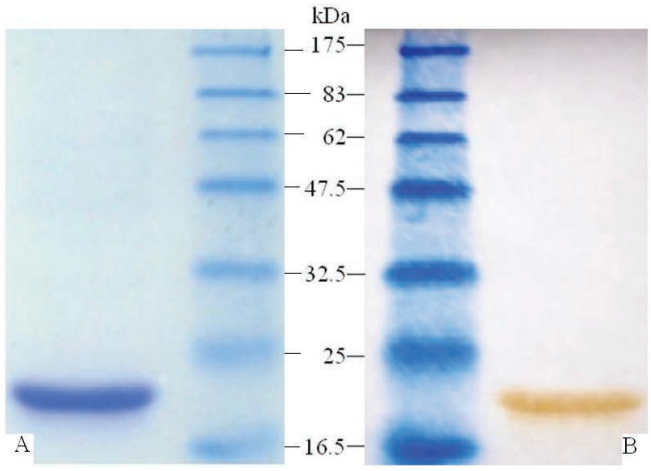

Figure 3: A. Or-bFGF was purified from *E.coli* using Ni-NTA. B. Purified protein was transferred to PVDF membrane and then probed with a monoclonal human bFGF antibody

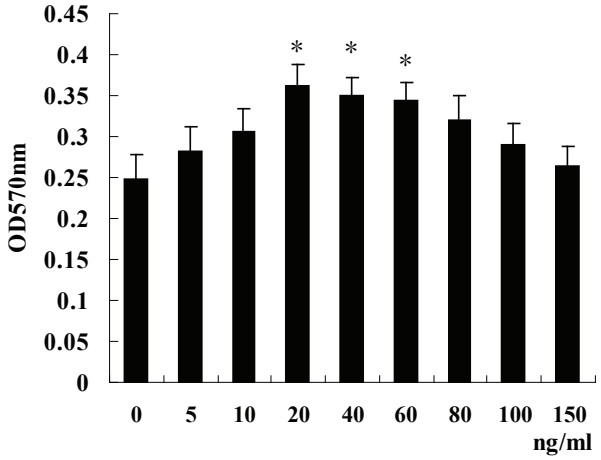

Figure 4: Effects of or-bFGF on proliferation of murine C2C12 myoblasts were detected with MTT assay. bFGF accelerated proliferation of C2C12 cells at the concentrations of 20, 40 and 60ng/ml (*P<0.05), whereas bFGF at other concentrations have no effects (P>0.05)

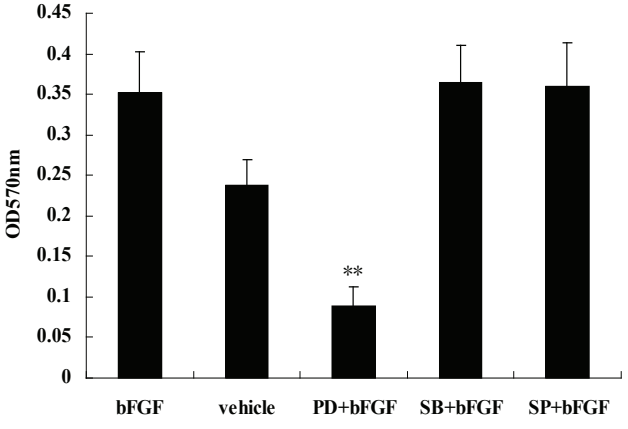

Figure 5: Effects of inhibitors of MAP kinase signaling pathway on proliferation of C2C12 myoblasts induced by bFGF.(PD:PD98059; SB:SB203580; SP:SP600125; **P<0.01, vs bFGF-stimulated cells)

Table 1: The effects of or-bFGF/hr-bFGF on growth of NIH-3T3 fibroblasts

Concentration (ng/ml)	OD570nm	
	or-bFGF	hr-bFGF
0	0.560±0.017	0.566±0.015
5	1.080±0.026	1.083±0.022
10	1.331±0.022	1.351±0.030
20	1.372±0.031	1.359±0.028
50	1.299±0.026	0.311±0.019
80	1.283±0.033	1.272±0.027
100	1.241±0.034	0.223±0.043

Effects of or-bFGF on proliferation of murine C2C12 myoblasts

As shown in Figure 4, the proliferation of murine C2C12 myobalsts were stimulated by or-bFGF significantly ($P<0.05$) at the concentrations of 20, 40 and 60ng/ml but insignificantly ($P>0.05$) at other concentrations. To determine the regulatory mechanisms, three inhibitors were employed respectively to block three MAPK signaling pathways. Results showed that PD98059 significantly suppressed proliferation of myoblasts induced by bFGF($P<0.01$), whereas SP600125 and SB203580 did not affect the proliferation significantly($P>0.05$, Figure 5), which demonstrated that bFGF employs MEK/ERK-MAPK signaling pathway, but not p38-MAPK and SAPK/JNK, to promote proliferation of myoblasts.

Discussion

Molecular characteristics and phylogenetic analysis

On the basis of our investigation, 18kDa-bFGF gene from Chinese Small Tail Han sheep carries most hallmarks attributed to the growth factor, such as receptor-binding sites, RGD sequences, phosphorylation sites and cysteine residues, these characters are important for functions of bFGF, they respectively contribute to binding receptors [20,21], modulation of mitogenicity [22], phosphorylation by protein kinase and forming an intramolecular disulfide bond to stabilize molecular structure [23], so the invariability of these key motifs is conducive to evolutional stabilization of bFGF gene. In addition, results of phylogenetic analysis showed that the growth factor gene remains highly conservation during evolution, the similarities of DNA or protein sequence between Small Tail Han sheep and other published terrestrial animals were found to be high, more than 84.8%, although the similarities between Small Tail Han sheep and amphibian or aquatic animals gradually decrease, but the similarities still keep a high percentage. By this token, bFGF is an evolutional conservative molecule and maybe fit to be regarded as a referenced molecule for biogeny.

Expression and activity assay of or-bFGF

Basic FGF is a growth factor that can stimulate proliferation of murine 3T3 fibroblasts. In this investigation, NIH-3T3 fibroblasts were used to determine biological activity of or-bFGF. In prokaryotic expression system, due to abundant expression, expressional products of exogenous gene probably form inclusion body to lose bioactivity, it is difficult to dissolve and renature the inactive protein. Therefore, to avoid plentiful inclusion body, appropriate conditions were selected and abundant soluble fusion protein was harvested. In addition, for fusion expression system, additional amino acid sequence fused to bFGF maybe affect activity of the target protein. So we detected activity of fusion pritein or-bFGF with NIH-3T3 cells. Results showed that His-Tag did not bring negative effects to bioactivity of the bFGF.

Roles of MEK/ERK-MAPK signaling pathway in proliferation of C2C12 myoblasts induced by bFGF

Basic FGF may be involved in skeletal muscle growth and differentiation by activating signaling pathways independent of PDGF-signaling pathways [24]. Skeletal muscle development and regeneration during embryonic and adult life consisting of proliferation, migration and differentiation of myogenic cells are regulated by several growth factors, in which bFGF has been confirmed to be an important growth factor for skeletal muscle regeneration [13,14,17,25]. Shishkin et al [26] reported that bFGF had no effects on proliferation of myoblasts at the concentrations of 20~80 ng/ml. However, in our experiments, or-bFGF at the concentration of 20~60ng/ml could significantly stimulate the cell proliferation, and or-bFGF at the other concentrations failed to affect proliferation of murine C2C12 myoblasts. As to the proliferation suppression of C2C12 myoblasts by high levels bFGF, it may result from that expression of fibroblast growth factor receptor was impaired by high concentration of exogenous bFGF.

Background researches have shown that bFGF employed different member/members of MAPK family to perform its biological roles in different cells[27-30]. As to skeletal muscle,

it is not clear that which member participates in the regulative mechanism. So we employed three inhibitors respectively to block three MAPK signaling pathways. Results showed that PD98059, inhibitor of MEK/ERK-MAPK signaling pathway, significantly suppressed proliferation of myoblasts induced by bFGF, whereas both of SP600125, inhibitor of SAPK/JNK-MAPK signaling pathway, and SB203580, inhibitor of p38-MAPK signaling pathway, did not affect the proliferation significantly, which demonstrated that bFGF employs MEK/ERK-MAPK signaling pathways, but not p38-MAPK and SAPK/JNK, to promote proliferation of myoblasts.

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KLONIRANJE BAZIČNEGA FIBROBLASTNEGA RASTNEGA DEJAVNIKA IZ KITAJSKE KRATKOREPE OVCE PASME HAN IN NJEGOVI UČINKI NA RAZMNOŽEVANJE MIŠJIH MIŠIČNIH CELIC C2C12

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Summary: Za boljše razumevanje lastnosti bazičnega fibroblastnega rastnega dejavnika (bFGF, FGF-2) in njegovih učinkov na skeletne mišice smo klonirali kodirajoče zaporedje 18kDa velikega gena bFGF iz kitajske kratkorepe ovce pasme han, odlične avtohtone pasme na Kitajskem. Zaporedje DNK in prevedena proteinska sekvenca sta bili analizirani in vzporejeni s homologi drugih živalskih vrst. Rezultati so pokazali, da je novo klonirani gen *bFGF* zelo podoben ostalim objavljenim zaporedjem bFGF pri drugih živalskih vrstah, kar nakazuje veliko evolucijsko ohranjenost gena bFGF. Popolno kodirajoče zaporedje gena *bFGF* je bilo vstavljeno v ekspresijski plazmid PET-28a iz česar je nastal fuzijski protein, izražen v *E. coli*. Rekombinantni protein je bil topen in biološko aktiven, primeren za nadaljnjo karakterizacijo. Preučili smo njegov vpliv na razmnoževanje mišjih mišičnih celic C2C12. Ugotovili smo, da ovčji rekombinantni bFGF pospeši njihovo razmnoževanje preko aktivacije signalne poti MEK/ERK-MAPK.

Ključne besede: bFGF; kloniranje; C2C12, MEK / ERK; kratkorepe ovce pasme han