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Amplification of rabbit hepatocyte growth factor and detection of its expression in COS-7 cell line

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Abstract

We used RT-PCR, nested PCR to acquire the partial 5′- race fragment of rabbit HGF cDNA and the partial 3′- race fragment of rabbit HGF cDNA. Then, we used recombination PCR to acquire rabbit HGF successfully. Homology analysis was conducted among the sequence of RABHGF and known human and rat HGF by DNAStar. It was proved that high level of homology existed among the sequences of those three HGF genes. We used the acquired gene of RABHGF to construct its recombinant eukaryotic expression vector pcDNA3.1(+)-RABHGF (pRABHGF). The identification of the eukaryotic expression vector pRABHGF by PCR, restriction enzyme and sequencing analysis showed that rabbit HGF gene was correctly inserted into the vector. pRABHGF and pcDNA3.1(+) as controls were transfected into COS-7 cells by lipofectamine. It takes 24h-36h after transfection to detect the expression of RABHGF protein by indirect immunofluorescence assay (IFA). The proliferation of cos-7 cells were evaluated by MTT assay. The result displayed positive effect of RABHGF protein on the proliferation of COS-7 cells. This study lays the foundation for a new gene therapy method for ischemic heart disease.

Key words: Hepatocyte growth factor, Nested PCR, Homology, Eukaryotic expression vector, Transfection.

Introduction

Michalopoulos et al (1) found a kind of material named hepatopoietin A in blood plasma of rat after partial hepatic resection to stimulate synthesis of hepatocyte DNA in 1984. That same year, one kind of hepatogenic factor isolated from blood plasma of rat after partial hepatic resection could stimulate primary cultured hepatocyte growth and DNA synthesis, so named hepatocyte growth factor (HGF) by Nakamura et al. (2), HGF is a kind of secretion glycoprotein, it had affinity for heparin, is also named scattering factor(SF). Human and rat HGF cDNA were cloned successfully by Nakamura in 1989 (3), Nakamura deducted their all amino acids sequence and discovered both have same structure. During the last decade, HGF was discovered in platelet of rats, blood serum of normal rats and patients after the extirpation of hepatocellular carcinoma, and plasma of the outbreak of liver failure patients. Further studies on the subject show that the same or very similar structure to above these material, recent published studies proved that rat and rabbit hepatocytopoiesis amino acid sequences are the same to human HGF gene sequence.

HGF has many biological functions. HGF has characteristics of apoptosis resistance, regulating cell growth and exercise, promoting occurrence of multiple cells morphology, anti-fibrosis effect, has important effect in embryo growth, tissue formation, liver regeneration and kidney regeneration, neoplasm metastasis and the repair of injury. HGF can promote proliferation of vascular endothelial cells, prevent coronary artery postoperative stenosis, prevent and treat arteriosclerosis. HGF has anti-fibrosis effect for the treatment of myocardial infarction (4,5). Nakamura etc (3). discovered HGF can promote mitogenic activity of vascular endothelial cells of human large arteries and rat coronary artery in vitro, but can not cause proliferation on vascular smooth muscle cells. Generally so called gene bypass grafting means introducing into cell growth factor or gene, it can improvement of blood supply in ischemic area, thus protects remnant myocytes and improves cardiac function (6,7).

We applied established experiment method based on rabbit model of chronic myocardial ischemia to study the new methods of treating myocardial infarction by applying HGF. Although there are some of cross activity between human, rat and rabbit HGF, it may acquire the highest activity by using homologous origin of rabbit HGF in rabbit model of chronic myocardial ischemia and induce the minimal side effects. Therefore, it is an optimal selection for rabbit model of chronic myocardial ischemia. Since the sequence of the rabbit HGF is still unknown, we designed the primers according to the conserved region of human and rat HGF nucleotide sequences. We amplified 5'-fragment of unknown rabbit HGF cDNA using the 5'RACE PCR. After sequencing of 5'-fragment of rabbit HGF cDNA, we designed the special upstream primers, amplified 3'-fragment of rabbit HGF cDNA by the 3'RACE PCR. Finally, we acquired the complete nucleotide sequences of rabbit HGF cDNA through applying recombination PCR. We used the acquired rabbit HGF to construct the recombinant eukaryotic expression vector pcDNA3.1(+)-RABHGF(pRABHGF). pRABHGF was transfected

into COS-7 cells by lipofectamine, and the expression of HGF protein on the transfected cells was observed by indirect immunofluorescence assay (IFA). MTT assay indicated the positive effect of HGF on the proliferation ratio of COS-7 cells.

Materials and methods

Reagent

Trizol reagent, M-MLV reverse transcriptase inhibitor, Taq DNA polymerase, pMD18-T vector and 3'-Full RACE Core Set Ver. 2.0, Endonuclease *Kpn* I and *Xba*l I were purchased from TakaRa. Plasmid kit was purchased from Tiangen. DNA gel extraction kit was from OMEGA. Goat polyclonal IgG HGFα(C-20), fetal bovine serum, RMPI 1640 culture solution, 0.25% trypsinase, and MTT were purchased from Santa Cruz Biotechnology. Lipofectamine 2000 was purchased from Life Technology. CCl_4 was purchased from Fluka. COS-7 cell line was obtained from ATCC. Others materials were provided by Harbin Veterinary Research Institue of Chinese Academy of Agricultural Scineces.

Animal

All procedures of animal handling were carried out in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the Harbin Medical University. Three Japanese White Rabbit (two female and one male) were provided by the Experimental Animal Center of Veterinary Research Institute in Heilongjiang Province.

Animal model of acute liver injury

Previous studies showed that HGF level is obviously increased in plasma after hepatotoxic agent injury (7, 8), thus we made acute liver injury rabbit model by feeding 2.5ml/kg CCl_4 through nose-stomach tube. CCl_4 was diluted 1:1 with corn oil. Control rabbits received corn oil alone. The rabbits were sacrificed 24 hours after the treatment of CCl_4.

ConstructionofpcDNA3.1(+)-rabbitHGF(pRABHGF)

Trizol reagent was used to extract total RNA from rabbit liver tissue and reverse transcription the RNA to cDNA by RT-PCR method [9]. Total RNA (2 μg each) was reverse transcribed. PCR conditions were optimized and linear amplification range was determined for each primer by varying annealing temperature and cycle number. Primers used are as follows:
upstream primer (U_1): 5′ ATGATGTGGGTGACCAAACT-3′, upstream primer(U_2): 5′ ATATCCCGACAAGGGCTTTGA-3′, upstream primer (U_3): 5′ CGCCAGCCCGTCCAGCAGCACC-3′, downstream primer (L_1): 5′-GGGAGCAGTAGCCAACTCGGA-3′,downstream primer (L_2): 5′-GTATTTCAAACTAACCATCCA-3′, downstream primer (L_3): 5′ CACGACCAGGAACAATGAC-3′.

DNA fragment was extracted from agarose gel by using gel extraction kit and acquire DNA fragment M_1 and M_2. The DNA fragment M_1 and M_2 was cloned into pMD18-T vector. The sequence of rabbit HGF was aligned with known human HGF and rat HGF, and analyzed by DNAStar.

Recombination PCR was done using DNA fragment M_1 and M_2 as templates, using upstream primer 5'-ATGATGTGGGTGACCAAACT-3', and downstream primer: 5'-GTATTTCAAACTAACCATCCA-3′. DNA fragment was extracted using gel extraction kit and DNA fragment was called as M_3. The DNA fragment M_3 was cloned into pMD18-T vector.

We designed upstream primers of outer and inner PCR according to the 5′-RACE fragment sequence of rabbit HGF: Upstream primer (W_1) 5′-GGGACAAGAACATGGAAGA-3′, Upstream primer (N_1): 5′-TGTGCCTTGGGATTATTGT-3′. Downstream primers of outer and inner PCR are provided by 3′-Full Race Core. The acquired DNA fragment was M_4. The DNA fragment M_4 was cloned into pMD18-T vector.

Recombination PCR was conducted as described above, using DNA fragment M3 and M4 as templates. Upstream primer (U4): 5′-GCGGATCCATGATGGGGGGGACCAAAC-3′-Downstream primer (L4): 5′-GCGTCTAGATTATGGCTGTGG CACCTTATACG-3′.

The two DNA fragment were connected with T_4 DNA Ligase.

COS-7 cell line culture and transfection

COS-7 cells were grown in RMPI 1640 culture medium plus 10% (vol/vol) fetal bovine serum, added with 100 μ g/ml penicillin-streptomycin. The cells were placed in 5% CO_2 humidified incubator at 37°C. Transfection was carried out using Lipofectamine2000. All procedures were followed by reagent manual.

Immunofluorescence assay

24h or 36h after transfection, primary antibody (goat anti-human HGF, Santa Cruz Biotechnology) was added into the cells, and incubated for 3h at room temperature. Then, secondary antibody (FITC-conjugated rabbit anti-goat IgG) was added. The slide was observed through inverted fluorescence microscope.

MTT assay

The transfected cells were incubated in 96-well plate for 24h, then add 20μl MTT solution (5mg/ml, 0.5% MTT) in each well. The cells were incubated at 37°C for 4 hours. Then, 100 μL of the SDS-HCl solution was added to each well and mix thoroughly using the pipette. The plate was incubated at 37°C for 4 hours in a humidified chamber. The absorbance was read at 540nm by a plate reader. Statistical analysis was conducted by SAS software. Paired t-test was used to determined statistic significance.

Results

PCR amplification

Agarose gel electrophoresis showed the bands M_1, M2, and M3 (Fig 1A-C). 3′- RACE PCR result indicated DNA fragment M_4 (Fig.1D). Recombination PCR results showed the full length of the rabbit HGF.

Confirmation by enzyme digestion

The DNA fragment M_3 was cloned into pMD18-T vector. The plasmid was identified by restriction enzyme digestion Sal I and *Bam*H I. (Fig 2A). Then, the DNA fragment M_4 was cloned into pMD18-T vector. The

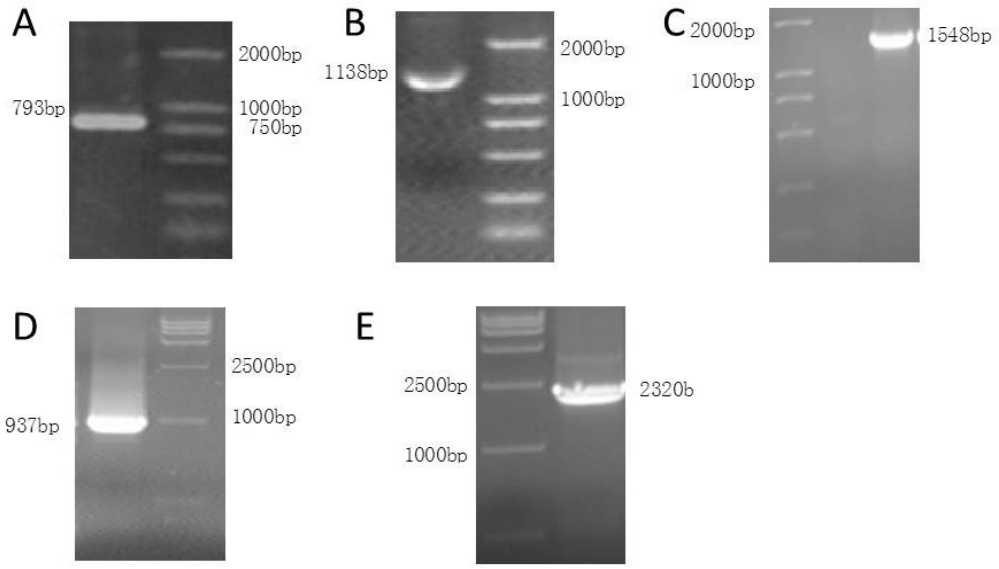

Figure 1. Electrophoresis result for PCR amplification. (A) AGE of M_1(793bp). (B) AGE of M_2(1138bp). (C) AGE of M_3(1548bp). (D) AGE of M_4(937bp). (E) AGE of the whole rabbit HGF cDNA(2320bp).

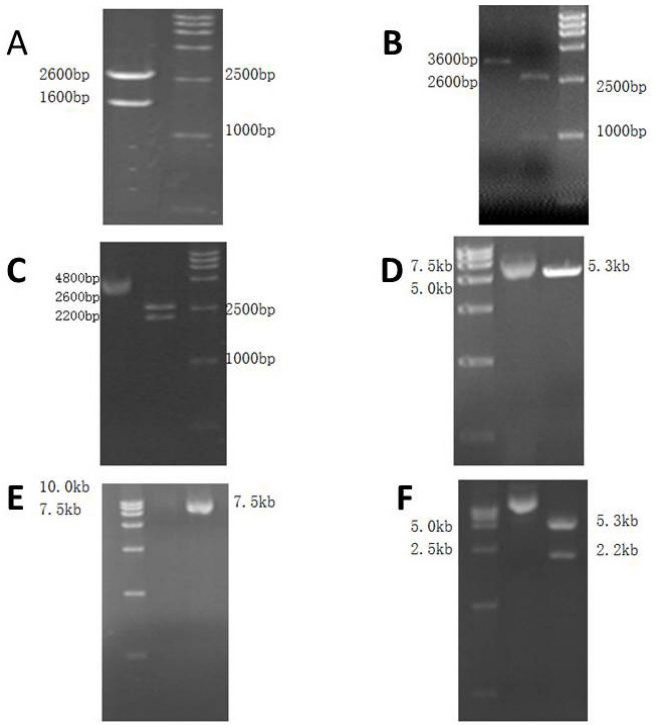

Figure 2. (A) AGE of pMD-T18-M3 by double restriction enzyme digestion Sal I and BamH I. (B) AGE of pMD-T18-M4 by restriction enzyme digestion BamH I. (C) AGE of pMD18-T-RABHGF (4800bp)Kpn I and Xba I. (D) AGE of pcDNA3.1(+) vector by Kpn I and XbaI. (E) AGE of pRABHGF. (F) AGE of pRABHGF by Kpn I and Xba I.

plasmid was identified by restriction enzyme digestion *Bam*H I (Fig.2B). The whole rabbit HGF was cloned into pMD18-T vector, and the plasmid RABHGF-pMD18-T was digested by double restriction enzyme digestion *Kpn* I and *Xba* I (Fig.2C). Electrophoresis was carried out to test the recombinant plasmid pRABHGF(Fig 2E). The recombinant plasmid pRABHGF was digested by *Kpn* I and *Xba* I and electrophoresis shows that two DNA fragments (Fig 2F).

Homology analysis

We acquired two kinds of the whole rabbit HGF cDNA sequence by sequencing of the recombinant plasmid pMD18-T-HGF. We named rabbit HGF-1(2299bp) and rabbit HGF-2(2320bp). The 21bp fragment was inserted into at the 854th base position of Rabbit HGF-2 cDNA. So the deduced amino acids sequence of rabbit HGF-2 are compared with rabbit HGF-1 inserted into 7 amino acids at the 285th base position.

The sequencing results of rabbit HGF were compared with known human HGF(2187bp) [GI:2171032] and rat HGF(2189bp) [GI:220437] sequence. Three kinds of gene sequences were carried out the homology analysis of nucleotide sequences and amino acids sequences through DNAStar. The homology is 89.3% of both nucleotide sequences after rabbit HGF-1 was compared with human HGF. The homology is 91.4% of the two deduced amino acids sequences. In addition, the homology is 86.8% of the nucleotide sequences of rabbit HGF-1and rat HGF. The homology is 92.0% of the two deduced amino acids sequences. Rabbit HGF-2 nucleotide sequences was compared with human HGF nucleotide sequences, we can find the homology is 89.1% both. The deduced amino acids sequences homology is 90.9%. Rabbit HGF-2 and rat HGF nucleotide sequences homology is 86.6%, the deduced amino acids sequences homology is 91.6%.

Expression of HGF on COS-7 cells

We observed FITC-labelled COS-7 cells 24h-36h after transfection of the pRABHGF (Fig.3A). The transfection efficiency was above 90%. COS-7 cells transfection pcDNA3.1(+) vector as control group did not show any fluorescence (Fig.3B).

RABHGF increased cell proliferation

The growth and proliferation of pRABHGF transfected COS-7 cells groups were compared with pcDNA3.1(+) vector transfected COS-7 cells control groups to detecting the function of rabbit HGF on cells proliferation by MTT. Three times independent experiments were carried out in the same experimental conditions. The statistic analysis showed that the absorbance was to 128% of control cells (Fig.4, $p<0.01$), showing clearly that recombinant rabbit HGF has the biological activity of promoting cell growth.

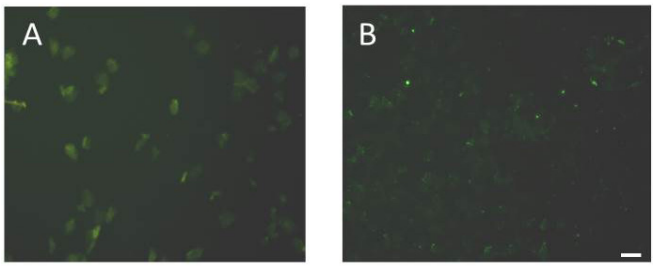

Figure 3. (A) pRABHGF transfected COS-7cells. (B) pcDNA 3.1(+) vector transfected COS-7cells. Scale bar: 20 μm.

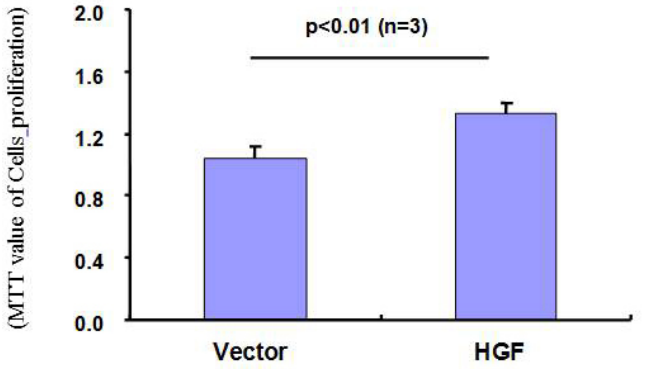

Figure 4. Detecting the effect of recombinant rabbit HGF on COS-7 cells proliferation by MTT assay.

Discussion

This experiment was designed studying the amplification of unknown gene sequence rabbit HGF cDNA. It is more difficult than know gene sequence amplification. Firstly, designing the primers must be reasonable and ensure the anticipant and successful results. We designed the primers according to the conservative region sequence of human HGF and rat HGF nucleotide sequences in GenBank. The partial 5′- RACE fragment of rabbit HGF was amplified and cloned, About the amplification of unknown gene sequence rabbit HGF cDNA, the cDNA template content was very low, it is difficult to acquire the anticipant and correct products applying the method of one-step PCR. Therebefore, we selected nested PCR to resolve this problem. The principle of 3′- RACE PCR is based on nested PCR principle, and use 3′- RACE PCR to amplify the 3′- RACE fragment of rabbit HGF. After acquiring the 5′- RACE fragment and 3′- RACE fragment of rabbit HGF. we selected recombination PCR to acquired the whole rabbit HGF cDNA.

We choose *Kpn* I and *Xba* I restriction site of eukaryotic expression vectors pcDNA3.1(+) and recombinant plasmid RABHGF-pMD18-T in the construction of eukaryotic expression plasmid pRABHGF of this experiment. We had suffered many times failures because of selection other restriction sites before Rabibt HGF was inserted into pcDNA3.1(+) vector. We modified primers and restriction site and eukaryotic expression plasmid pRABHGF was construced successfully finally. Indirect immunofluorescence assay was performed in the transient transfection of pRABHGF recombinant plasmid in stable eukaryotic expression COS-7 cells by liposome method at 24h and 36h. The results of indirect immunofluorescence assay showed that COS-7 cells expressed and secreted rabbit HGF protein.

Various transcripts of HGF mRNA were found by comparing the different HGF cDNA cloning. They caused different varieties of HGF because of different splicing (10). There have the fuction of promoting hepatocyte growth and proliferation in the reported different mammalian HGF, but they have big difference at the aspects of source, molecular weight and properties. We amplified two kinds of Rabbbit HGF gene named Rabbbit HGF-1 and Rabbbit HGF-2 respectively in our laboratory. Rabbbit HGF-1 cDNA sequences contains 2299 nucleotides encoding 762 amino acids, Rabbbit HGF-2 cDNA sequences contains 2320 nucleotides encoding 769 amino acids. The 21 nucleotides fragment (GAAGCCGTTATTTTGCAAGAG) were insert into at the 854th position of Rabbbit HGF-2 cDNA sequences, so there are 7 amino acids (GSRYFAR) inserted into the 285th base position of rabbit HGF-2 compared with rabbit HGF-1, but nucleotide sequences and amino acids sequences are same at other position. The longest open reading frame of Rabbbit HGF-1 is same as that of Rabbbit HGF-2.

Our experiment results showed that Rabbbit HGF genes were amplified and pRABHGF recombinant plasmid was construced successfully. The secretory expression of Rabbbit HGF protein has the biological activity of promoting cells growth in Rabbbit HGF gene transfection COS-7 cells. Established rabbits myocardial ischemia model and Rabbbit HGF gene were used to study further research on gene therapy of ischemic heart disease. It will play a great role in the field of cardiovascular medicine with the development of HGF to the depth. We develop a new gene therapy method in ischemic heart disease.

In a word, the more deeply research of HGF, researchers pay attention to its functions in the fields of cardiology to supply a new way to clinical diagnosis and treatment in future. A new gene therapy way applied HGF for vascular reconstruction, which lays the foundation for subsequent experimental study through this experiment.

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References

1. Nakamura, T., Nawa, K., and Ichihara, A., Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem. Biophys. Res. Commun.* 1984, **122**: 1450-1459.

2. Nakamura, T., Nishizawa, T., Hagiya M, Seki, T., Shimonishi, M., Sugimura, A., Tashiro, K., and Shimizu, S., Molecular cloning and expression of human hepatocyte growth factor. *Nature* 1989, **342**: 440-443. doi: 10.1038/342440a0

3. Duan, H. F., Wu, C. T., Wu, D. L., Lu, Y., Liu, H. J., Ha, X. Q., Zhang, Q. W., Wang, H., Jia, X. X., and Wang, L. S., Treatment of myocardial ischemia with bone marrow- derived mesenchymal stem cells overexpressing hepatocyte growth factor. *Mol. Ther.* 2003, **8**:467- 474.

4. Khan, T. A., Sellke, F. W., and Laham, R. J., Gene therapy progress and prospects: therapeutic angiogenesis for limb and myocardial ischemia. *Gene Ther.* 2003, **10**: 285- 291. doi: 10.1038/sj.gt.3301969.

5. Tahlil, O., Brami, M., Feldman, L. J., Branellec, D., and Steg, P. G., The dispatch catheter as a delivery tool for arterial gene transfer. *Cardiovasc. Res.* 1997, **33**:181-187. doi: 10.1016/S0008-6363(96)00188-5.

6. Rosengart, T. K., Lee, L. Y., Patel, S. R., Sanborn, T. A., Parikh, M., Bergman, G. W., Hachamovitch, R., Szulc, M., Kligfield, P. D., Okin, P. M., Hahn, R. T., Devereux, R. B., Post, M. R., Hackett, N. R., Foster, T., Grasso, T. M., Lesser, M. L., Isom, O. W., and Crystal, R. G., Angiogenesis gene therapy : phase I assessment of direct intramyocardial administration of an adenovirus vector expressing VEGF121 CDNA to individuals with clinically significant severe coronary artery disease. *Circulation* 1999, **100**: 468 - 474.

7. Lindroos, P.M., Zarnegar, R., and Michalopoulos, G.K., Hepatocyte growth-factor (hepatopoietin-a) rapidly increases in plasma before dna-synthesis and liver-regeneration stimulated by partial-hepatectomy and carbon-tetrachloride administration. *Hepatology* 1991, **13**: 743-749. doi: 10.1016/0270-9139(91)92574-R.

8. Shiota, G., Okano, J., Kawasaki, H., Kawamoto, T., and Nakamura, T., Serum hepatocyte growth factor levels in liver diseases: clinical implications. *Hepatology* 1995, **21**: 106-112.

9. Bozkurt, S., Ersoy, E., Tekyn, H. E., Bayram, O., Anadol, Z., Onuk, E., and Ercan, Z. S., The cytoprotective effect of iloprost against carbon tetrachloride induced necrosis in rat liver. *Res. Commun. Mol. Pathol. Pharmacol*. 1997, **95**: 343-346.

10. Yu, X., He, X., Jiang, J., He, J., Fan, R., Wang, H., Geng, J., and Dong, C., Expression and tissue distribution of hepatocyte growth factor (HGF) and its receptor (c-Met) in alpacas (Vicugna pacos) skins associated with white and brown coat colors. *Acta. Histochem.* 2015, **117**: 624-628. doi: 10.1016/j.acthis.2015.06.002.