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Molecular cloning, expression, and functional features of *IGF1* splice variants in sheep

Xu-Ting Song[1,*], Jia-Nan Zhang[1,*], Duo-Wei Zhao[1], Yu-Fei Zhai[1], Qi Lu[1], Mei-Yu Qi[2], Ming-Hai Lu[3], Shou-Long Deng[4], Hong-Bing Han[5], Xiu-Qin Yang[1] and **Yu-Chang Yao[1]**

[1]Key Laboratory of Animal Genetics, Breeding and Reproduction, Education Department of Heilongjiang Province, College of Animal Science and Technology, Northeast Agricultural University, Harbin, China
[2]Institute of Animal Husbandry, Heilongjiang Academy of Agricultural Sciences, Harbin, China
[3]Department of Animal Science, Heilongjiang State Farms Science Technology Vocational College, Harbin, China
[4]CAS Key Laboratory of Genome Sciences and Information, Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing, China
[5]Beijing Key Laboratory of Animal Genetic Improvement, China Agricultural University, Beijing, China

Correspondence should be addressed to Y-C Yao or X-Q Yang: yaoyc@neau.edu.cn or xiuqin163@163.com

*(X-T Song and J-N Zhang contributed equally to this work)

Abstract

Insulin-like growth factor 1 (IGF1), also known as somatomedin C, is essential for the regulation of animal growth and development. In many species, the *IGF1* gene can be alternatively spliced into multiple transcripts, encoding different pre-pro-IGF1 proteins. However, the exact alternative splicing patterns of IGF1 and the sequence information of different splice variants in sheep are still unclear. In this study, four splice variants (class 1-Ea, class 1-Eb, class 2-Ea, and class 2-Eb) were obtained, but no *IGF1 Ec*, similar to that found in other species, was discovered. Bioinformatics analysis showed that the four splice variants shared the same mature peptide (70 amino acids) and possessed distinct signal peptides and E peptides. Tissue expression analysis indicated that the four splice variants were broadly expressed in all tested tissues and were most abundantly expressed in the liver. In most tissues and stages, the expression of class 1-Ea was highest, and the expression of other splice variants was low. Overall, levels of the four *IGF1* splice variants at the fetal and lamb stages were higher than those at the adult stage. Overexpression of the four splice variants significantly increased fibroblast proliferation and inhibited apoptosis ($P < 0.05$). In contrast, silencing *IGF1 Ea* or *IGF1 Eb* with siRNA significantly inhibited proliferation and promoted apoptosis ($P < 0.05$). Among the four splice variants, class 1-Ea had a more evident effect on cell proliferation and apoptosis. In summary, the four ovine *IGF1* splice variants have different structures and expression patterns and might have different biological functions.

Key Words
- sheep
- *IGF1*
- splice variants
- tissue expression
- proliferation
- apoptosis

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Introduction

Insulin-like growth factor 1 (IGF1), also known as somatomedin C, has remarkable diversity in terms of biological effects. It is widely recognized that IGF1 is essential for the regulation of normal growth (1), development (2), immunity (3), and metabolism (4) in vertebrate species. The important role of IGF1 in growth and development makes it a promising candidate gene for the marker-assisted selection of growth traits (5).

Alternative splicing, occurring in over 95% of human genes and over 63% of mouse genes, plays key roles in the regulation of gene expression and diversification of both the transcriptome and encoded proteome (6, 7). *IGF1* is conserved across species and contains six exons that can

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be alternatively spliced into multiple transcripts, encoding different pre-pro-IGF1 proteins (8, 9, 10). The IGF1 proteins are named according to differences in the leader and carboxy-terminus (8). Briefly, according to differences in the leader, the splice variants starting from exon 1 or exon 2 are referred to as class 1 or class 2 IGF1, respectively. Meanwhile, according to the difference in the carboxy-terminus, the splice variants containing exon 5 or exon 6 are generally referred to as IGF1 Eb or IGF1 Ea, respectively. Moreover, the splice variant containing both exons 5 and 6 is referred to as IGF1 Ec (also known as mechano growth factor). Finally, this alternative splicing leads to different signal peptides and E peptides of the IGF1 protein domains.

Different splice variants of the *IGF1* gene have different expression patterns and various functions (11, 12). Class 1 transcripts are widely expressed in all tissues with relatively high levels, whereas class 2 transcripts are expressed in only a few tissues with low levels, except in the liver (8, 11). It has been proposed that class 1 transcripts might be associated with an autocrine/paracrine role and have a stronger ability to interact with insulin-like growth factor-binding proteins (13), whereas class 2 transcripts represent the endocrine form and are thought to be more growth hormone (GH)-dependent (14, 15). More recently, the roles of the E peptide in regulating IGF1 function have come under scrutiny, especially with respect to myoblast proliferation and muscle repair. During skeletal muscle injury, IGF1 Ea is responsible for satellite cell differentiation, whereas IGF1 Ec is responsible for satellite cell activation and proliferation (16, 17).

Different splicing patterns of IGF1 in humans and rodents have been reported previously (8, 18, 19). However, the exact splicing patterns of IGF1 and the sequence in formation of different splice variants are still unclear in sheep. In this study, full-length coding sequences of different *IGF1* splice variants were acquired from the ovine liver, and the expression levels of different splice variants were quantified in 12 tissues of sheep at different ages. Then, their effects on cell proliferation and apoptosis were analyzed using skin-derived primary fibroblasts. The objectives of our study were to investigate IGF1 alternative splicing patterns and analyze the functional features of these variants in sheep.

Materials and methods

Ethics statement

All sheep were managed under normal husbandry conditions. All experimental animal protocols were approved and performed in accordance with the requirements of the Animal Care and Use Committee at Northeast Agricultural University (approval ID 2016–067).

Animals and tissue collection

Northeast fine-wool sheep were utilized in this study and housed in the breeding base of Northeast Agricultural University (Harbin, China). Northeast fine-wool sheep is a native meat-wool breed in the northeast of China. In our study, nine animals were selected at 20 days pre-birth and 30 days and 36 months of age as fetal, lamb, and adult groups (three sheep per group), respectively. The liver, lung, spleen, heart, kidney, bladder, intestine, stomach, skin, testis, adipose tissue, and leg muscle were collected. All tissues were obtained within approximately 15 min, immediately frozen in liquid nitrogen, and subsequently stored at −80°C. Additionally, the skin-derived primary fibroblasts were isolated and cultured from a fetal lamb at 20 days pre-birth.

Total RNA isolation and cDNA synthesis

Total RNA was extracted using the TRIzol reagent (Takara) according to the manufacturer's protocol. The ratios of absorbance at 230, 260, and 280 nm were used to evaluate the purity and concentration of total RNA using the NanoDrop spectrophotometer (NanoDrop ND-1000, Wilmington, DE, USA). Then, the integrity of total RNA was assessed by 1.2% (w/v) agarose gel electrophoresis, and samples showing good RNA quality were selected for further RT. Total RNA (1 μg) was used for cDNA synthesis with PrimeScript RT reagent with gDNA Erase kit (Takara) in accordance with the manufacturer's instructions. The products were stored at −20°C until use.

Cloning and bioinformatics analysis of sheep *IGF1* splice variants

Based on GenBank and Ensemble sequences of *IGF1* splice variants in different species, a conserved region was found by aligning sequences from multiple species. Considering sequence specificity and structural integrity, four pairs of primers were designed to amplify full-length coding sequences of different splice variants. Additionally, according to species homology, the *Sus scrofa IGF1 Ec* transcript was used as a reference to determine whether *Ovis aries IGF1 Ec* exists. Based on the characteristics of *IGF1 Ec* (with insertion of the sectional exon 5 sequence

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into the junction of exon 4 and exon 6) and the conserved sequences of the entire *IGF1* gene among different species, we designed one forward primer and three reverse primers to amplify pig and sheep *IGF1 Ec*. The partial exon 5 (*IGF1 Ec* transcript specified) was amplified from the liver and muscle of sheep. All primers are listed with their sequences in Supplementary Table 1 (see section on supplementary materials given at the end of this article). With the primers, the full-length sequences of the *IGF1* splice variants were amplified using PCR from the cDNA of the ovine liver. The PCR mixture contained 2.5 μL of 10 × r Taq buffer (TaKaRa), 1 μL of cDNA, 1 μL of dNTP (10 mM, Takara), 1 μL of 0.25 μmol/L forward primers, 1 μL of 0.25 μmol/L reverse primers, 18 μL of H_2O, and 0.5 μL r Taq DNA polymerase (Takara). The amplification conditions were pre-denaturing at 94°C for 5 min; 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 7 min. All the PCR products were detected by agarose gel electrophoresis and recovered using an Agarose Gel DNA Purification Kit (TIANGEN, Beijing, China). The products were cloned by pMD18-T vector (Takara) and sequenced (GENEWIZ, Nanjing, China).

The open reading frames (ORFs) of *IGF1* splice variants of sheep were analyzed and translated into amino acid sequences. The potential signal peptide was predicted with SignalP 4.0. Multiple alignment of amino acid sequences was performed using DNAman (Lynnon Biosoft, Canada).

IGF1 splice variants expression

We analyzed *IGF1* expression using semi-quantitative PCR mainly due to the special splicing pattern of the *IGF1* gene, whose splicing sites simultaneously locate at exon 1/2 and 5/6. To distinguish the four splice variants (class 1-Ea, class 1-Eb, class 2-Ea and class 2-Eb), two splicing sites should be simultaneously analyzed. Thus, a 417 bp fragment, at least, which contain exon 1 (63 bp)/2 (15 bp), exon 3 (160 bp), exon 4 (182 bp), and exon 5 (162 bp) /6 (60 bp), must be amplified using qPCR. However, the amplification efficiency of enzyme in the qPCR reagents is difficult to meet the sensitive and quantitative measurement of such a long fragment (below 200nt) (20). Moreover, the probe method is still unable to effectively distinguish the four splice variants due to the existence of two splicing sites at the same time. Therefore, the relatively reasonable and stable semi-quantitative PCR was selected as the main experimental method to quantify all of the four splice variants. All primers were designed based on the sequences obtained in this study. The information of the primers for the four *IGF1* splice variants, as well as the reference gene (β-*actin*), are displayed in Supplementary Table 1. All of the target sequences were initially sequenced to ensure the specificity of the primers.

The composition of the PCR mix reaction was described above. The PCR protocol consisted of pre-denaturation at 94°C for 5 min, followed by the main 32 cycles (denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 40 s), which was determined by gradient cycles from 26 to 38, and a final extension at 72°C for 10 min. Then, 5 μL of PCR products from splice variants and 5 μL of the β-*actin* products were mixed and electrophoresed on a 2.5% agarose gel. The ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) was used to detect gray values. All samples, including the non-template control, were run in triplicate and mean values were subsequently used for analysis.

Construction of expression plasmids and selection of siRNA sequences

The expression plasmids of *IGF1* splice variants were constructed based on the EGFP-pcDNA 3.1 vectors (YRBIO, Shanghai, China). The coding sequences (CDSs) of the four *IGF1* splice variants were amplified using the ovine liver cDNA as a template, and then, the target fragments were cloned into the vectors using specific primers. The specific primers were designed to contain sequences for HindIII and EcoR I restriction enzymes (underlined; Takara). These plasmids were designated as pcDNA 3.1-1 Ea, pcDNA 3.1-1 Eb, pcDNA 3.1-2 Ea, and pcDNA 3.1-2 Eb.

Potential target siRNA sequences for sheep *IGF1* splice variants were screened using a siRNA target finder program (http://RNAiDesigner.invitrogen.com). The siRNA sequences for *IGF1 Ea* and the negative control were synthesized by GenePharma Co (Shanghai GenePharma Co, Shanghai, China). Negative control siRNA consisted of the same length of 21 nucleotides without homology to any sheep gene sequence.

Cell culture

The skin-derived primary fibroblasts were isolated from fetal tissue and cultured according to the method of Zhang *et al.* (21). Briefly, fibroblasts were purified by the differential adherent method. The cells were cultured in high Glucose-Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS) (Gibco BRL) at 37°C with 5% CO_2. The cells used in our experiments were not passaged more than eight times.

Transfection of plasmids and siRNA

The cells were seeded in six-well plates containing antibiotic-free medium and subjected to transfection when they reached 70% confluency. Then, the plasmids or siRNA were transfected into cells with lipofectamine 2000 (Invitrogen) and RfectPM transfection reagent (BIOG, Shenzhen, China) according to the manufacturers' respective protocol. Non-transfected cells were used as control cells. The transfection rates of expression plasmids were assessed in the presence of green fluorescent protein (GFP) using a fluorescent microscope. The interference rates of the specific siRNAs were assessed using semi-quantitative PCR and Western blotting.

Western blotting

The cells were washed twice with PBS and lysed with RIPA buffer (Beyotime, Shanghai, China) supplemented with a proteinase inhibitor. Protein concentrations were determined using the Enhanced BCA Protein Assay kit (Beyotime). Then, 40 μg of total protein sample was treated at 95°C for 10 min after being resuspended in 5× sodium dodecyl sulfate (SDS) loading buffer and was separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked in 5% skim milk for 2 h at 37°C and then incubated at 4°C overnight with anti-IGF1 antibody (Bioss, Wuhan, China) and anti-β-actin antibody (Cell Signaling). After being washed three times with tris buffered saline with tween (TBST), the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Beyotime) at 20–25°C for 1 h. Following three additional washes with TBST, the membrane was developed using an ECL kit (Beyotime). The gray value of each band was determined using the ImageJ software (National Institutes of Health; version 1.45). The relative expression of proteins was expressed as the ratios of target protein band intensities to those of β-actin.

Cell proliferation assay

Cell proliferation analysis was carried out using cell counting kit-8 (CCK-8) according to the manufacturer's protocol (Beyotime). Briefly, the cells were transiently transfected with plasmids or siRNAs as described and then seeded in 96-well plates and tested at 24, 48, and 72 h. Next, 10 μL of CCK-8 solution was added to each well, and the samples were incubated for 4 h before the absorbance was measured at 450 nm using a microplate.

Cell apoptosis assay

Cell apoptosis analysis was carried out using an Annexin V-PE/7DD apoptosis kit according to the manufacturer's protocol (KeyGen, Jiangsu, China). Briefly, the cells were transfected with plasmids or siRNAs as described and then seeded in six-well plates and tested at 48 h. The cells were washed three times with phosphate-buffered saline (PBS) and 55 μL of the 7-ADD solution was added to the harvested cells in the dark for 15 min. Then, 450 μL of binding buffer and 1 μL Annexin V-PE were added to the cells, followed by a 15 min incubation. The fluorescence was analyzed using a FACS Calibur (BD Biosciences, San Jose, CA, USA) and the FlowJo software (FlowJo, LLC, Ashland, OR, USA).

Statistical analysis

Statistical analysis was performed with SPSS software 18.0 (SPSS Inc.). All data are presented as the mean ± S.E.M. Differences in mRNA and protein expression, cell proliferation, and apoptosis were analyzed using a one-way ANOVA procedure, followed by Duncan's *post hoc* test. Differences in the tissue expression of *IGF1* transcripts were analyzed using a general linear model procedure, followed by Duncan's *post hoc* test. Statistical significance was defined as $P < 0.05$.

Results

Cloning of sheep *IGF1* splice variants

The full-length CDSs of four *IGF1* splice variants were successfully cloned in this study. The four fragments (Fig. 1A) obtained were 534, 610, 485, and 561 bp and were designated as class 1-Ea, class 1-Eb, class 2-Ea, and class 2-Eb, respectively. BLAST searches in NCBI Genbank among species indicated that the four transcripts acquired in this study were *IGF1* sequences from sheep. The sequence of class 1-Ea has been previously reported (NM_001009774.3). The new splice variants (class 1-Eb, class 2-Ea, and class 2-Eb) found in this study have been uploaded to Genbank (GenBank accession no. MG572781, MG572782, and MG572783).

We detected the presence of the *IGF1 Ec* gene in pigs, which is the darker fragment in Fig. 1B. However, these fragments were not obtained in sheep. Therefore, the *IGF1 Ec* gene in sheep, predicted from other species, was not found in this study.

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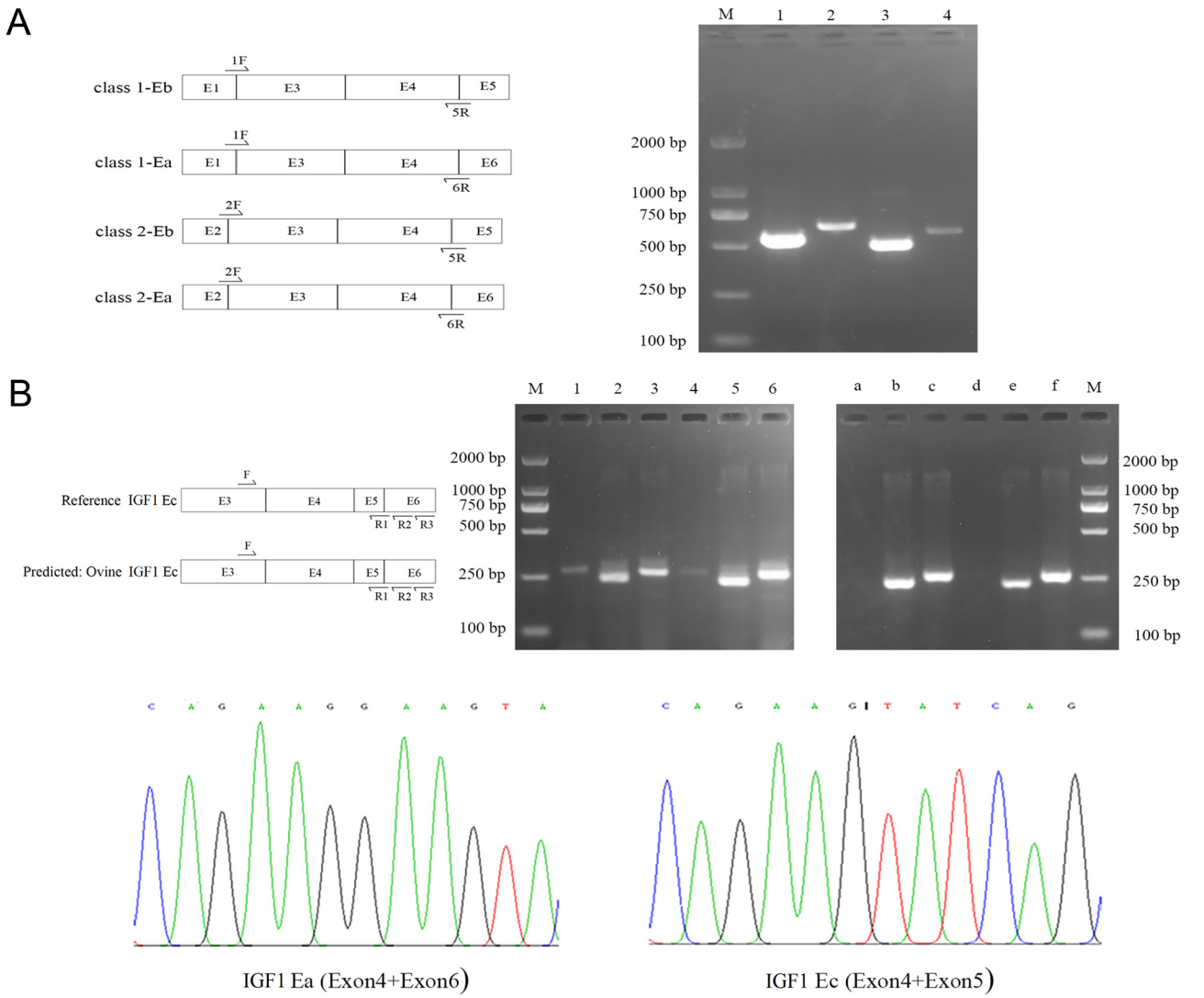

Figure 1 Cloning of *IGF1* splice variants in sheep. (A) Locations of primers and agarose gel electrophoresis of amplified PCR products from four *IGF1* splice variants. PCR primers are indicated by arrows. Lane M: marker DL 2000. Lane 1 to Lane 4: PCR products of class 1-Ea, class 1-Eb, class 2-Ea and class 2-Eb. (B) Locations of primers, agarose gel electrophoresis, and peak figure of amplified PCR products from *IGF1 Ec* in pig and sheep. PCR primers are indicated by arrow. The PCR products of the lane 1–3 and 4–6 are from porcine liver and muscle, respectively. The PCR products of Lane a–c and d–f are from ovine liver and muscle, respectively. Lane M: marker DL 2000. Lane 1, 4 and the upper band of Lane 2, 3, 5, 6 are pig *IGF1 Ec*. The lower band of Lane 2, 3, 5, 6 and lane b, c, e, f are pig and sheep *IGF1 Ea*, respectively.

Sequence analysis of sheep *IGF1* splice variants

Alignment with the corresponding cDNA sequences revealed complex structure for sheep *IGF1* mRNA, which consisted of six exons (Fig. 2A) and had typical structural domains of the *IGF1* gene (signal peptide, mature peptide, and E peptide). The ORF lengths of class 1-Ea, class 1-Eb, class 2-Ea, and class 2-Eb were 465, 567, 417, and 519 bp (Fig. 2B), respectively, encoding 154, 188, 138, and 172 amino acids, respectively. All splice variants encoded the same mature peptide of 70 amino acids (Fig. 2C). An analysis of mature peptides among nine species indicated that the sheep IGF1 mature peptide contained four different amino acid residues compared to that in rodents but only one different amino acid residue at position 66 compared to that of seven other species. Potential signal peptide cleavage sites were indicated at amino acids 49–50 for class 1 IGF1 and amino acids 33–34 for class 2 IGF1 (Fig. 2D). These results showed that different transcripts have distinct nucleic acid sequences, amino acid sequences, ORFs, signal peptides, and E peptides (Table 1).

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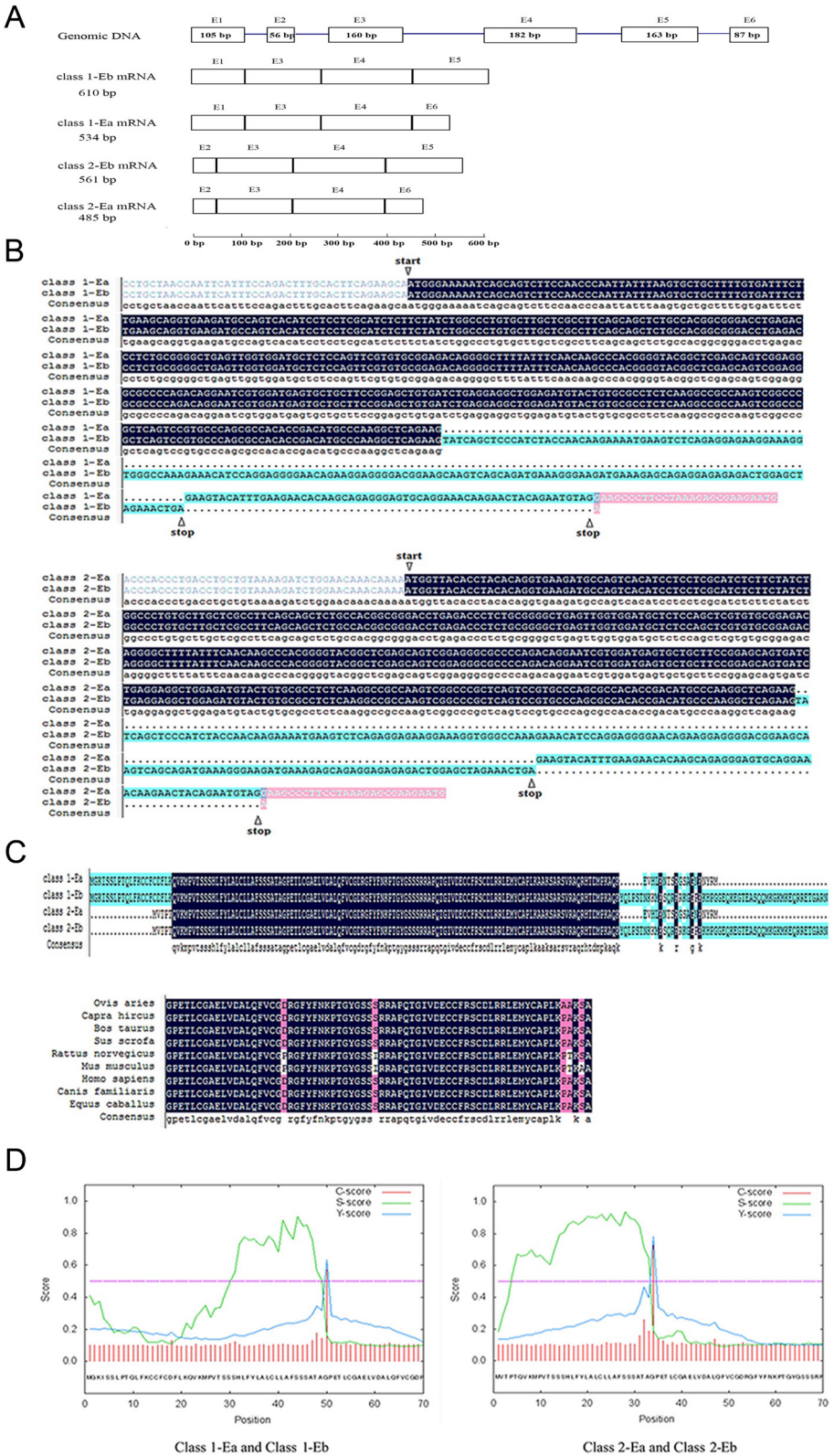

Figure 2

Nucleotide and amino acid sequences of four IGF1 splice variants. Inverted triangle marked as translation initiation codon and terminal codon. (A) Structure of four *IGF1* splice variants. These mRNAs have been deposited into the Genbank database. (B) Nucleotide sequences of splice variants obtained by sequencing. (C) Multiple alignments of IGF1 mature amino acid sequences among nine mammals. (D) Signal peptide for two class splice variants.

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Table 1 Sequence analysis of four *IGF1* splice variants in sheep.

Name	Composition	Length	ORF	Protein	Signal peptide	Mature protein	E peptide
Class 1-Ea	Exons 1–3–4–6	534	465	154	49	70	35
Class 1-Eb	Exons 1–3–4–5	610	567	188	49	70	69
Class 2-Ea	Exons 2–3–4–6	485	417	138	33	70	35
Class 2-Eb	Exons 2–3–4–5	561	519	172	33	70	69

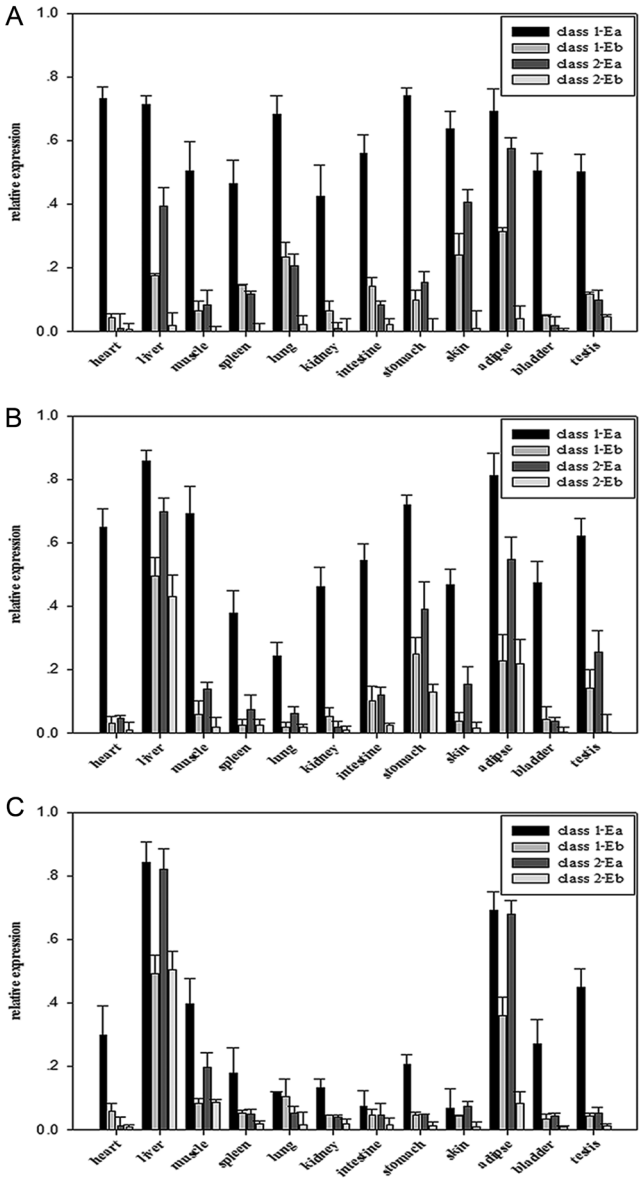

Figure 3
Expression levels of four splice variants at three stages. (A) Expression levels of four splice variants in fetal sheep (n = 3). (B) Expression levels of four splice variants in lamb sheep (n = 3). (C) Expression levels of four splice variants in adult sheep (n = 3).

Expression levels of the four splice variants in different tissues and stages

We next examined the expression levels of the four *IGF1* splice variants in 12 tissues at different ages in sheep. As shown in Fig. 3 and Supplementary Fig. 1, the expression patterns of the four *IGF1* transcripts were distinct in various tissues but similar in different stages. In all tissues, the expression level of class 1-Ea mRNA was highest and the expression of class 2-Eb mRNA was low at most stages.

Additionally, we compared the expression levels of the four transcripts in the same tissue at different stages. In most cases, levels of the four *IGF1* splice variants at the fetal and lamb stages were higher than those at the adult stage. In the heart, spleen, lung, and skin, the highest expression levels of the four splice variants were at the fetal stage, when compared to those at the other developmental stages, in which expression was downregulated. At the fetal stage, class 1-Ea was most abundantly expressed, and this was expressed at a significantly higher level compared to that at the adult stage in the heart ($P < 0.01$), lung ($P < 0.05$), and skin ($P < 0.01$; Figs 4A and 5C). Simultaneously, although the expression of class 1-Ea in the six aforementioned tissues at the lamb stage was lower than that at the fetal stage, it was still significantly higher than that at the adult stage. Moreover, class 1-Eb and class 2-Ea also exhibited significantly higher expression levels at the fetal stage than at the adult stage in the lung ($P < 0.05$) and skin ($P < 0.05$; Figs 4E and 5C).

However, the expression levels of these four splice variants peaked in the liver, muscle, kidney, bladder, stomach, and testis at the lamb stage. Class 1-Ea was most abundantly expressed at the lamb stage, showing significantly higher expression levels compared to those in the adult stage in the kidney ($P < 0.05$) and testis ($P < 0.05$; Figs 4F and 5F). Both class 1-Eb and class 2-Ea exhibited significantly higher expression levels at the lamb stage than at the adult stage in the stomach ($P < 0.01$) and testis ($P < 0.05$; Fig. 6B and F). However, class 2-Eb was not differentially expressed at different developmental stages in all tissues, except for in the stomach (the expression level at the lamb stage was significantly higher than that at the fetal and adult stage; $P < 0.05$).

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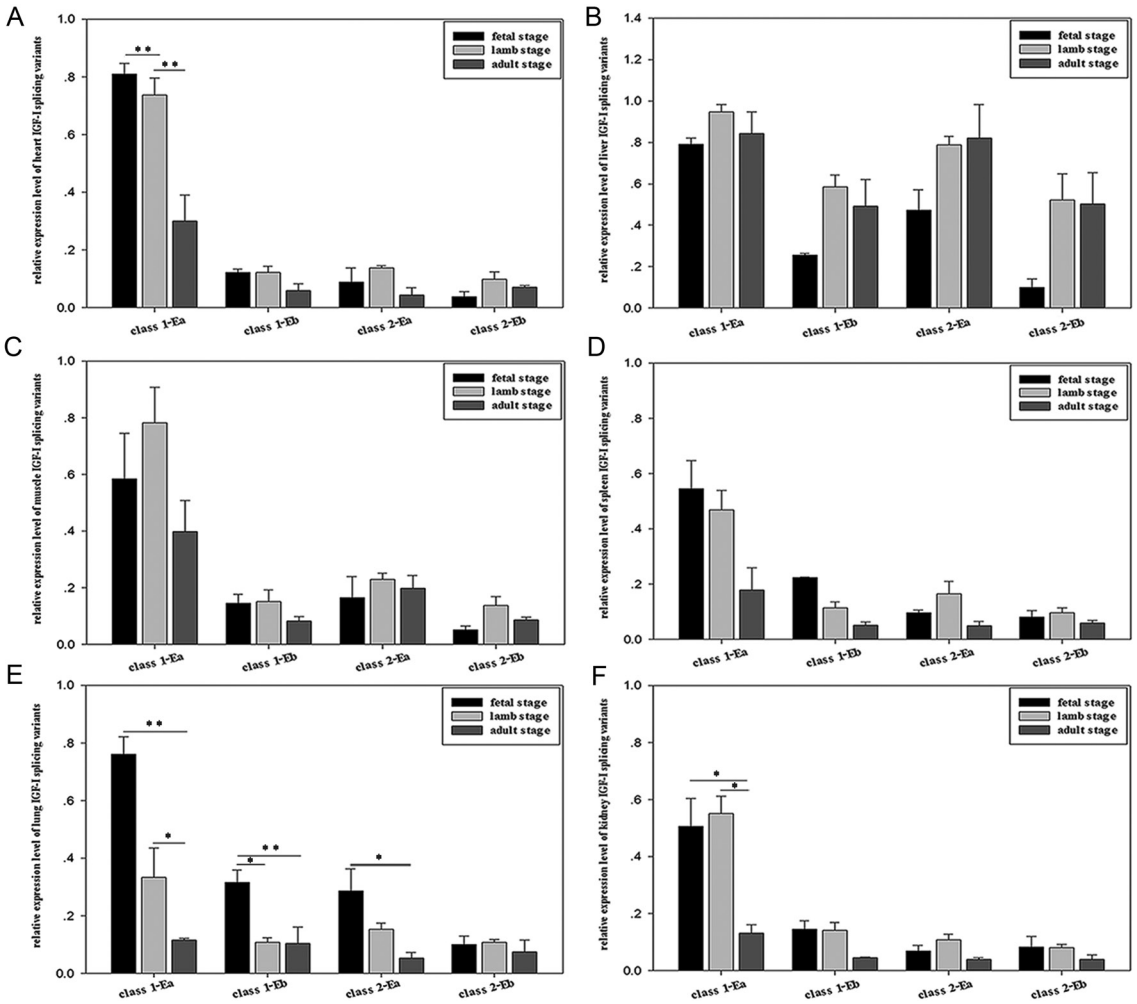

Figure 4
Expression levels of four splice variants in different tissues. (A, B, C, D, E and F) Expression levels of four splice variants in the ovine heart, liver, muscle, spleen, lung and kidney tissue. Differences were considered to be statistically significant when $P < 0.05$ (*) and $P < 0.01$ (**).

Overexpression and silencing of *IGF1* splice variants in primary fibroblasts

Overexpression and RNA interference were performed by transfecting primary fibroblasts with plasmids expressing *IGF1* splice variants or specific siRNA, respectively. After 48 h of transfection, PCR and Western blotting were performed to determine the mRNA and protein expression levels of IGF1 splice variants. As shown in Fig. 6A, expression levels of the four IGF1 splice variants at the mRNA and protein levels were all significantly increased after overexpression ($P < 0.01$). In contrast, silencing IGF1 Ea and IGF1 Eb significantly inhibited the mRNA and protein expression of both genes ($P < 0.01$, Fig. 6B).

Overexpression and silencing of *IGF1* splice variants alters cell proliferation

Furthermore, we investigated the effect of overexpressing or silencing *IGF1* splice variants on cell proliferation. As shown in Fig. 7A, overexpression of all four *IGF1* splice variants decreased cell proliferation significantly compared to that with pcDNA 3.1-NC or the control ($P < 0.05$). Class 1-Ea overexpression had the strongest ability to promote cell proliferation and was associated with the largest absorbance value compared to that with the other splice variants ($P < 0.01$). In contrast, silencing IGF1 Ea or IGF1 Eb had inhibitory effects on cell proliferation ($P < 0.05$), and proliferation was largely inhibited after IGF1 Ea was silenced ($P < 0.05$, Fig. 7B).

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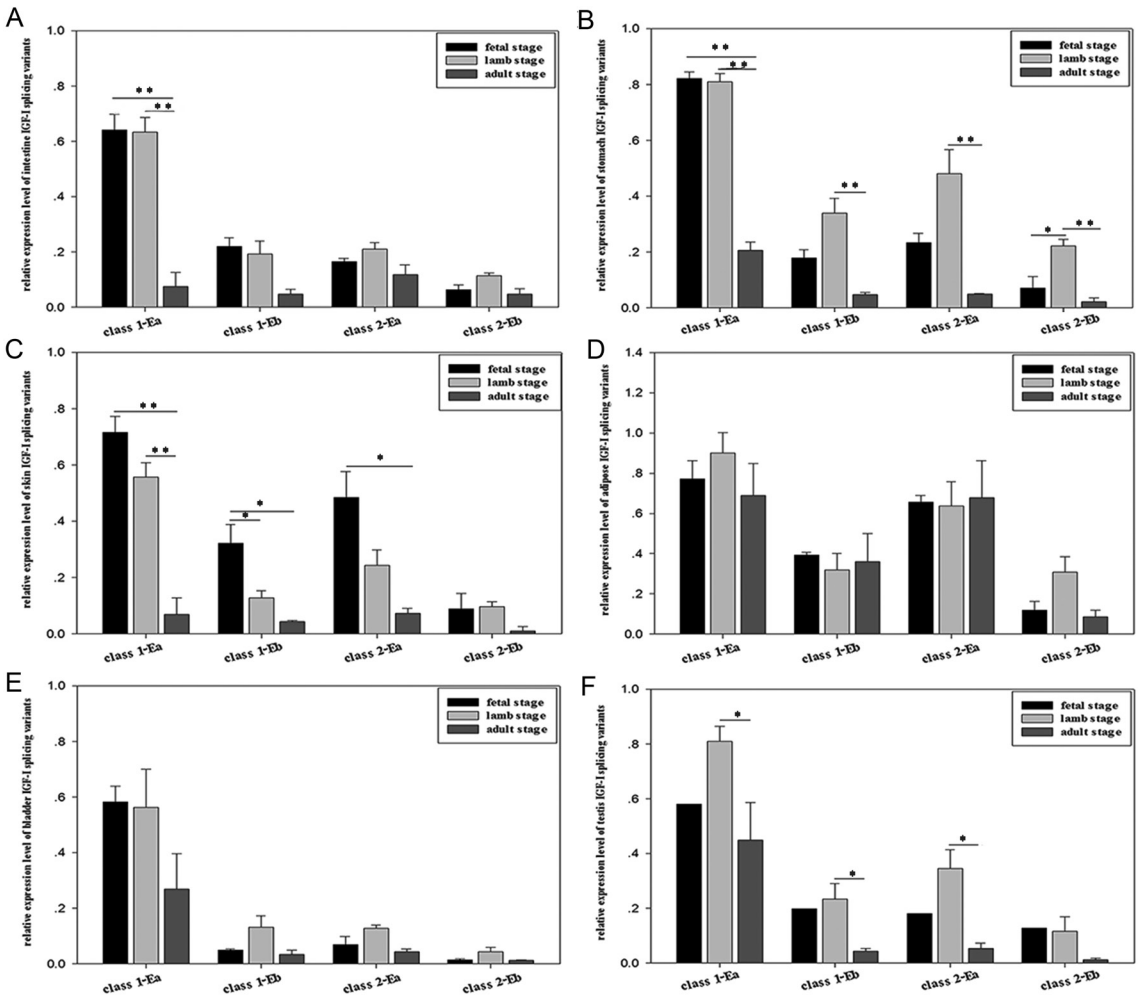

Figure 5
Expression levels of four splice variants in different tissues. (A, B, C, D, E and F) Expression levels of four splice variants in the ovine intestine, stomach, skin, adipose, bladder and testis tissue. Differences were considered to be statistically significant when $P < 0.05$ (*) and $P < 0.01$ (**).

Overexpression and silencing of *IGF1* splice variants alters cell apoptosis

Finally, we determined the effect of *IGF1* splice variant overexpression or silencing on cell apoptosis. Based on our results, both the overexpression and silencing of *IGF1* splice variants significantly influenced apoptosis. As shown in Fig. 7B, overexpression of the four *IGF1* splice variants decreased the apoptosis rate significantly compared to that with pcDNA 3.1-NC or the control ($P < 0.05$). Similar to the results of cell proliferation, class 1-Ea overexpression also had the strongest ability to inhibit cell apoptosis relative to that with other splice variants. In contrast, silencing IGF1 Ea or IGF1 Eb led to an increase in cell apoptosis ($P < 0.05$), and the downregulation of IGF1 Ea had a greater effect on apoptosis than inhibiting IGF1 Eb.

Discussion

Alternative splicing can increase the complexity of gene expression and cause transcripts from the same pre-mRNA to have diverse biological functions (10). In this study, four *IGF1* splice variants, class 1-Ea, class 1-Eb, class 2-Ea, and class 2-Eb, were acquired from the ovine liver. Bioinformatics analysis revealed that all four splice variants had three typical structural domains, including a signal peptide, mature peptide, and E peptide. They also shared the same mature peptide of 70 amino acids and had different signal peptides and E peptides.

The mature peptide of IGF1 with biological activity is responsible for binding to its receptors (22). An analysis of the mature peptides of 70 amino acids among nine species indicated that they were highly conserved with those of seven other species but not rodents. The sheep IGF1 mature

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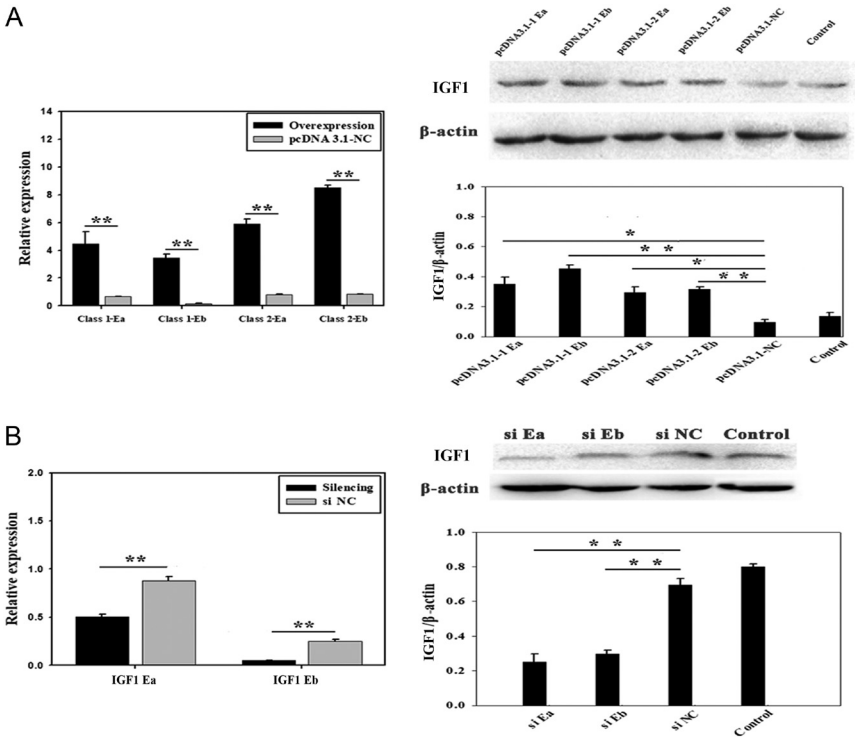

Figure 6
The mRNA and protein expression of IGF1 splice variants in sheep primary fibroblasts transfected with expression plasmids or specific siRNA. (A) The mRNA and protein expression of IGF1 splice variants after transfection with expression plasmids. (B) The mRNA and protein expression of of IGF1 Ea and Eb after transfection with specific siRNA. Differences were considered to be statistically significant when $P < 0.05$ (*) and $P < 0.01$ (**).

peptide contained only one different amino acid residue at position 66 (Fig. 2C), as compared with the sequences of these seven species. This amino acid change at the end of the mature peptide had no effect on the highly conserved unique pentabasic prohormone cleavage motif, which can lead to the cleavage of pro-IGF1 through the removal of the carboxy-terminus E peptide (22). Such high conservation of the mature peptides corresponding to different IGF1 transcripts across multi-species could ensure stable binding to IGF1 receptors.

Although IGF1 is thought to exert its biological actions predominantly through the mature peptide, diverse biological activities have been reported for the splice variants with different signal peptides or E peptides (23, 24). The signal peptide located at the N-terminus is cleaved during endoplasmic reticulum translation, which is considered a key component controlling cellular secretion and the targeting of proteins and pro-peptides (25). Our results showed that the four *IGF1* splice variants of sheep possess two types of signal peptides with different lengths and compositions. Class 1 transcripts possessed a longer signal peptide (49 amino acids in length) compared to class 2 transcripts (33 amino acids in length). The class 2 transcripts have a higher secretion efficiency due to the presence of typical signal peptide motifs (26, 27). Moreover, class 2 transcripts are thought to be more GH-dependent, because they do not structurally contain the DNase I hypersensitive site (28, 29). A previous study has shown that the expression of hepatic class 2 transcripts is upregulated and two-fold higher than class 1 transcript expression with a single injection of GH in cattle (11).

The E peptides, located at the carboxy-terminus, are obtained by proteolytic cleavage of the pro-protein convertases on the pro-IGF1 and control IGF1 production and secretion by preventing systemic circulation (30, 31, 32). The E peptides from IGF1 splice variants share less than 50% amino acid identity among different species (19, 33). Our results revealed that there are two E peptides (Ea and Eb) in sheep IGF1, depending on the presence of exon 6 or exon 5. Ea and Eb were found to contain only 16 common amino acids, accounting for 45% of total IGF1 Ea amino acids and 23% of the total IGF1 Eb amino acids. Further, IGF1 Ea contained the N-linked glycosylation site based on the Asn-X-Ser/Thr sequence, whereas Eb lacked this site due to the reading frameshift. This N-linked glycosylation could regulate intracellular IGF1 Ea levels by preventing proteasome-mediated degradation (34, 35).

Meanwhile, IGF1 Ec has been found in humans (19), rats (36), and pigs (10), and its roles in muscle repair and regeneration have been the focus of recent studies. By aligning *IGF1 Ec* sequences of the aforementioned three species, the splicing pattern of *IGF1 Ec* was found

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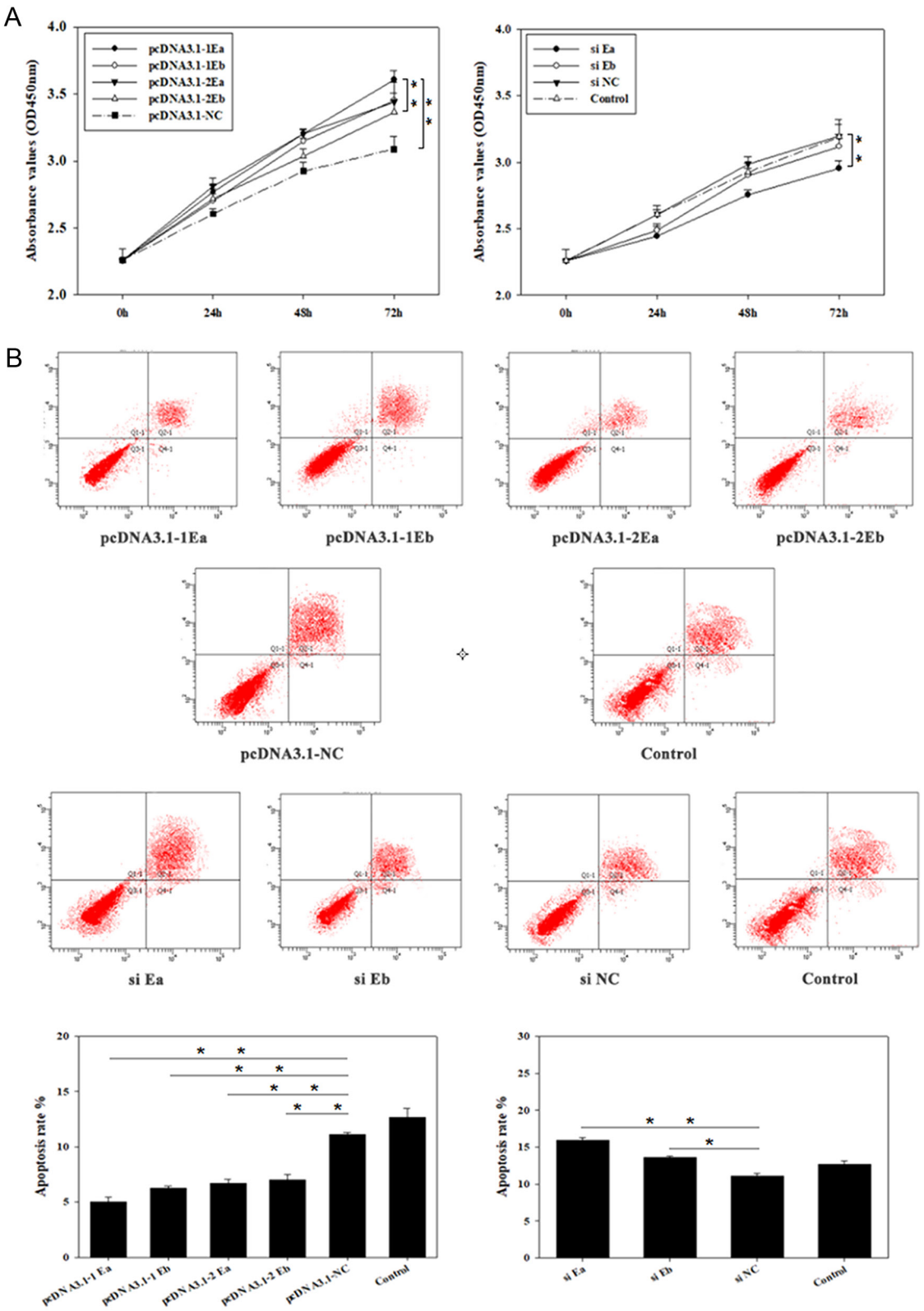

Figure 7

Change of the proliferation and apoptosis of sheep fibroblasts after up- and downregulation of IGF1 splice variants expression. (A) The fibroblasts proliferation after transfection with expression plasmids or specific siRNA. (B) The fibroblasts apoptosis rates after transfection with expression plasmids and specific siRNA. Differences were considered to be statistically significant when $P < 0.05$ (*) and $P < 0.01$ (**).

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to be highly conserved among species. In our study, the predicted sheep *IGF1 Ec* sequence was amplified according to the conserved region. We failed to obtain sheep *IGF1 Ec*, although Giosuè Annibalini *et al.* reported that *IGF1 Ec* does exist in sheep (37). Some researchers have speculated that *IGF1 Ec* is only expressed in damaged or loaded skeletal muscle (38, 39). Therefore, under normal physiological conditions, sheep *IGF1 Ec* might not be expressed, which is different from that in pigs. Thus, the presence of *IGF1 Ec* in sheep needs to be further verified.

Although IGF1 is widely expressed in all tissues of most species, the expression patterns of its splice variants are relatively restricted and change with growth and development. In this study, we found that the four splice variants of sheep were ubiquitously expressed in all tissues. Class 1-Ea was the primarily expressed transcript, whereas the other transcripts were expressed at relatively low levels. The signal peptide of class 1-Ea is not a typical signal peptide sequence, but a longer mammalian signal peptide containing a myristylation site, which is key to maintaining normal circulating IGF1 (40). Persistently high expression of hepatic class 1-Ea might provide basal levels of IGF1 for normal growth and development (10). Therefore, due to the compensatory upregulation of class 1 transcripts, the knockout of class 2 transcripts in mice has no effect on the circulation of IGF1 and on animal growth and development (15). Approximately, 75% of the circulating IGF1 originates from the liver, which is the major source of this protein (41, 42). Other transcripts in the liver at all stages were relatively less expressed; for example, class 2-Ea expression was only ~20% of class 1 Ea expression, which is consistent with the results in mouse and heifer studies (8, 43). Furthermore, the expression of hepatic class 2 transcripts is upregulated after birth. Some studies have shown that increased expression of class 2 *IGF1* mRNA might be a mechanism used by animals to achieve accelerated growth (10, 12, 44). Although it is a classic circulating hormone, IGF1, produced by autocrine and paracrine patterns, still plays an important regulatory role (10, 12, 42). Similar to the results in cattle (11) and pig (10), a variety of sheep tissues also express these four *IGF1* splice variants, such as skin, heart, kidney, fat, lung, rumen, small intestine, spleen, and testis. However, in mice, the class 2 transcripts are not expressed in the heart, brain, and muscle (10, 12, 45). Interestingly, the expression of IGF1 transcripts in almost all tissues at the adult stage was lower than that at fetal and lamb stages, possibly due to a decrease in the responsiveness of the promoter after rapid growth and development (26). IGF1 is central to the GH-IGF1 somatotrophic axis, in which its synthesis is regulated by the pituitary secretion of GH (46). GH regulated the *IGF1* promoter region through JAK/STAT pathway with activated Stat proteins (47). It was reported that the GH signaling was downregulated in the adult and could delay aging (48). These results indicate that the expression patterns of different IGF1 transcripts are related to their structure and exhibit spatial and temporal regularity.

IGF1, as an important mitogenic and anti-apoptotic regulator, performs essential roles in animal growth (49, 50). Complete deletion of the *IGF1* gene in mice results in death or survival with severe growth defects (51). After overexpressing the rat *IGF1* gene in the liver of *IGF1*-null mice, the transgene was found to maintain normal post-natal growth by elevating circulating IGF1 levels (52). *In vitro* IGF1 has been shown to be associated with cell proliferation and apoptosis in various cells, such as mesenchymal progenitor cells, intimal smooth muscle cells, and porcine kidney cells (53, 54). In this study, we found that the overexpression of any of the four IGF1 splice variants significantly increased cell proliferation and inhibited apoptosis ($P < 0.01$). Among these splice variants, class 1 Ea had a more evident effect on cell proliferation and apoptosis because of its higher baseline expression level. In contrast, silencing of IGF1 Ea or IGF1 Eb inhibited cell proliferation and induced apoptosis, and the effect of IGF1 Ea was more obvious than that of Eb. Similar to our results, interfering with IGF1 Ea in myoblasts reduces cell proliferation, and furthermore, treatment with mature IGF1 can restore Myh3 expression to control levels (55). The general consensus is that the mature IGF1 could effectively activate IGF1R after the C-terminal of E peptides is removed. Both forms of E peptides (Ea and Eb) in a free state could still act as the regulator of mature IGF1 (56) and affect cell proliferation and apoptosis by modulating IGF1/IGF1R signaling (57, 58). The E-peptides cooperating with IGF1 could increase IGF1R phosphorylation, localization, and downstream signaling, this process was due to the augmentation of IGF1R phosphorylation through increasing the proportion of receptors on the cell surface (59). We speculated that the difference between IGF1 Ea and Eb in promoting proliferation and inhibiting apoptosis might be caused by the presence of the specific sequences within E peptides, such as the N-linked glycosylation site, thereby changing the activation efficiency of IGF1R (56). Taken together, these results support the contention that the IGF1 splice variants are involved in cell proliferation and apoptosis.

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In the present study, the four *IGF1* splice variants were acquired from the ovine liver, but IGF1 Ec was not obtained. Bioinformatics analysis showed that all transcripts have typical structural domains of the *IGF1* gene but different signal peptide motifs and E-peptides. Expression analysis indicated that the four *IGF1* splice variants have different spatial and temporal expression patterns. At most development stages, the expression level of class 1-Ea was highest and the expression of class 2-Eb was low. Up- or downregulation of the four *IGF1* splice variants affected the proliferation and apoptosis of fibroblasts. Among these splice variants, class 1 Ea had a more evident effect on cell proliferation and apoptosis. Therefore, the four ovine *IGF1* splice variants have different structures and expression patterns and might have different biological functions.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/EC-21-0181.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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