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A synonymous mutation in *IGF-1* impacts the transcription and translation process of gene expression

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Insulin-like growth factor 1 (IGF-1) is considered to be a crucial gene in the animal development of bone and body size. In this study, a unique synonymous mutation (c.258 A > G) of the *IGF-1* gene was modified with an adenine base editor to observe the growth and developmental situation of mutant mice. Significant expression differences and molecular mechanisms among vectors with different alanine synonymous codons were explored. Although modification of a single synonymous codon rarely interferes with animal phenotypes, we observed that the expression and secretion of IGF-1 were different between 8-week-old homozygous (Ho) and wild-type (WT) mice. In addition, the IGF-1 with optimal codon combinations showed a higher expression content than other codon combination modes at both transcription and translation levels and performed proliferation promotion. The gene stability and translation initiation efficiency also changed significantly. Our findings illustrated that the synonymous mutation altered the *IGF-1* gene expression in individual mice and suggested that the synonymous mutation affected the *IGF-1* expression and biological function through the transcription and translation processes.

INTRODUCTION

Insulin-like growth factor 1 (IGF-1), as an active growth factor regulating a variety of metabolic processes, more importantly, is regarded as one of the major promotors during animal growth and development.[1,2] Researchers found IGF-1-deficient mice showed growth retardation and reduced bone structure and demonstrated IGF-1 acts from birth to puberty and leads to the proportional size increase in a variety of tissues.[3,4] On the other hand, it is reported that in the majority of mammals, DNA sequence conservation is moderately high in homologous IGF-1 exons and proximal promoter regions.[5,6] In terms of these performances, Cheng et al.[7,8] have found only one synonymous mutation *IGF-1* c.258 A > G(rs322131043) in the *IGF-1* between two pig breeds with opposite growth rates and body sizes, which is located in the coding region for binding to its receptor IGF-1R. Thus, given the high conservation in *IGF-1* sequences and its amino acid composition, the positive selection of this synonymous mutation in animal evolution may be an indispensable driving force for the dwarfism formation in miniature pigs.[9]

Evidence now indicates synonymous mutations are shaped by evolutionary selection and affect other aspects of protein biogenesis beyond specifying the amino acid sequence of the protein including transcription, translation, co-translation folding, secretion, and post-translation modification, although they were once thought to be functionally neutral.[10–12] Additionally, a significant difference was preliminarily proven in *IGF-1* gene-expression levels, *IGF-1* gene stabilities, and protein-binding affinity, with its receptor between AA and GG genotypes by Cheng et al.[7,8]. However, the studies of Cheng et al.[7,8] were also only limited to the use of expression vectors to report changes in gene expression *in vitro*. Similarly, most current basic research on synonymous mutations remains at the cellular level.[13] Few examples of *in vivo* experiments have been used to examine the contribution of synonymous mutations to biological phenomena or attempt to assess the impact of these mutations broadly and comprehensively. Consequently, although the synonymous mutation identified above has been retained by genetic selection and is likely to have some potential biological functions, further verification is still expected by the method of base editing model animals here, and the research on synonymous codon regulation mechanisms of a specific gene also needs to be carried out.

Unlike traditional methods to introduce point mutations, base editors are relatively accurate means to convert a single base at the present stage, enabling targeted direct, irreversible single-nucleotide modification in genomic DNA without requiring double-stranded DNA (dsDNA) breaks (DSBs), homology-directed repair (HDR) processes,

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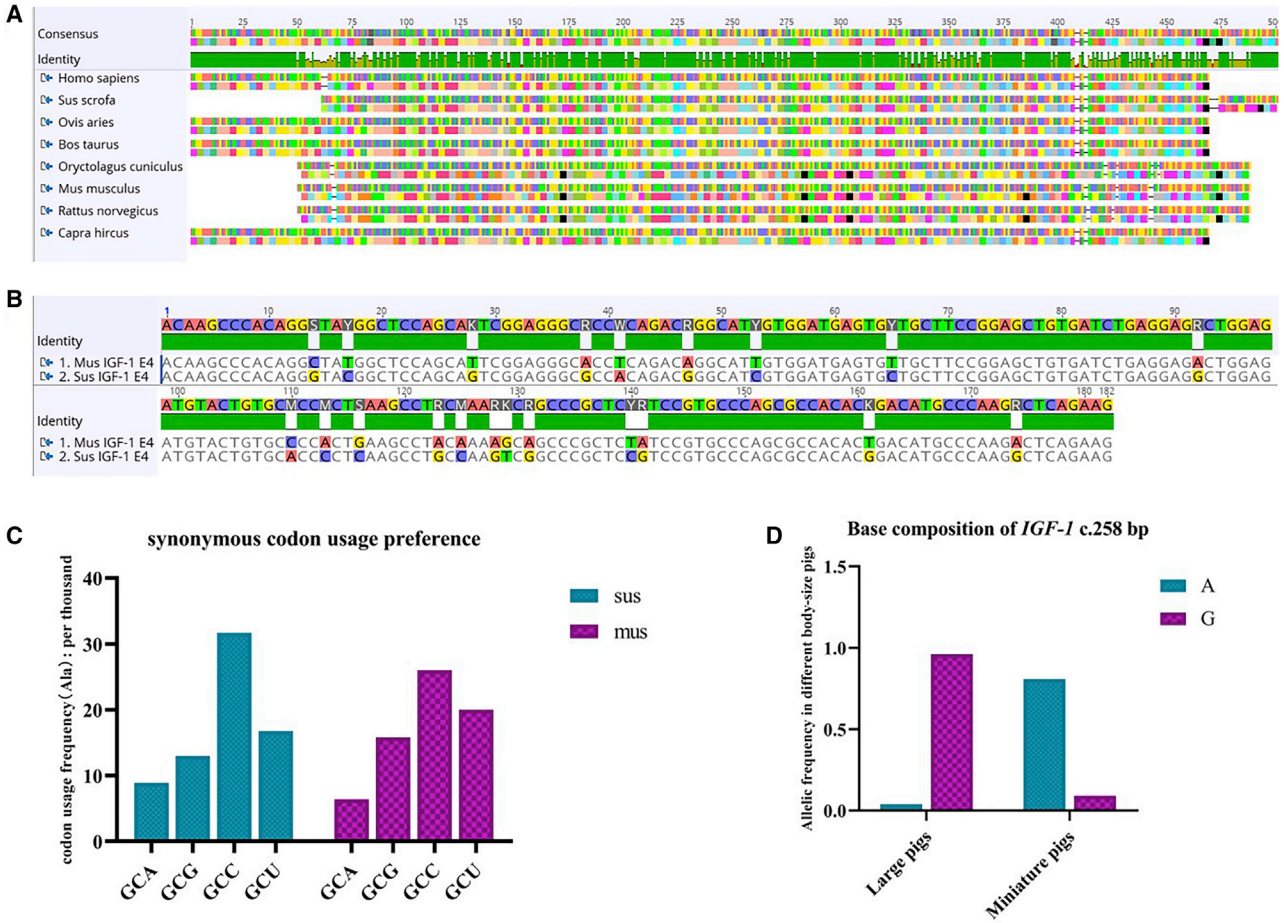

Figure 1. Bioinformatics analysis of *IGF-1* gene homology and distribution of codon preference for the synonymous mutation site *IGF-1* c.258bp

(A and B) Homology alignments of IGF-1 mRNA sequences among different species (A) and *IGF-1* exon 4 in Sus scrofa and *Mus musculus* (B). The four base types are distinguished by different colors, and the green part indicates sequence consistency. (C) Synonymous codon usage frequency of Ala in Sus scrofa and *Mus musculus*. (D) The frequency of *IGF-1* c.258 A > G among pigs with different body size.

or a donor DNA template.[14,15] Furthermore, base editors can proceed more efficiently and reduce undesired products to ensure the safety of editing.[16–18] In order to achieve the adenosine (A) - to-guanosine (G) conversion, adenine base editors (ABEs) were developed and have undergone diverse improvement to substantially increase the base-editing utility. To date, the ABEmax system, as one of the latest versions of ABEs, has been successfully applied to modify missense mutations in mammals,[14,18–22] which also provided a precedent for the study of synonymous mutations in animals.

Therefore, the purpose of this study was to clarify the influence of genetic diversity on gene biosynthesis and secretion based on the potentially meaningful *IGF-1* c.258 A > G synonymous mutation screened. Here, the mutational mice with *IGF-1* c.258 A > G were introduced by ABEmax. Results demonstrated that *IGF-1* c.258 A > G induced the fluctuation of gene expression and secretion *in vivo*, although no significant characters of miniature stature and abnormal bone development were observed. In this research, we reveal for the first time the effect of a single synonymous single-nucleotide polymorphism (SNP) at the individual animal level and explore how a synonymous codon influences the biological function of a gene through both transcription and translation processes *in vivo*. Taken together, these results provide novel insights into the role of the *IGF-1* synonymous mutation in gene-expression regulation and further supply new data for the formation mechanism of miniature pigs.

RESULTS

IGF-1 synonymous codons perform a conservative preference

To investigate whether *IGF-1* synonymous mutations have potential regulatory mechanisms for animal body-type traits, first, a fairly high level of organizational and nucleotide sequence similarity among 25 different species in the *IGF-1* gene was evaluated,[5] and homology analysis revealed that IGF-1 was highly conserved, especially in humans, mice, rats, pigs, and rabbits (Figure 1A). Subsequently, 88.46% of percentage identity was shown by comparing the homology of the exon 4 of *IGF-1* in pigs and mice, where the

Table 1. The allelic frequency of *IGF-1* c.258 A > G among pigs with different body size

	Large pigs (n = 180)		Miniature pigs (n = 42)	
Alleles	A	G	A	G
Individuals	7	173	34	8
Frequencies	3.89%	96.11%	80.95%	9.05%

Table 2. Distribution of *IGF-1* c.258 A > G allele preference among different mammalian species

Body types	Species	Number	Preference
Short stature	mice	48	A
	rabbit	2	A
	dog	67	A
Large stature	horse	72	G
	cattle	98	G
	goat	235	G
	sheep	129	G

synonymous codon c.258 A > G is located (Figure 1B). For the majority of amino acids, synonymous codons were largely encoded by optimal or non-optimal codons, while possessing different regulatory properties. Research has integrated a heatmap showing the codon stability coefficient (CSC) sorted by the encoded amino acid and found that for alanine (Ala), the contribution of different synonymous codons to mRNA stability and expression was discrepant in diverse species.[23] Therefore, we calculated the codon usage frequency of Ala in pigs and mice, and the results suggested that although the frequency has slightly deviated, there was a relatively consistent preference. Of the four synonymous codons, GCC is the most frequently used, followed by GCU and GCG, whereas GCA has the lowest usage frequency (Figure 1C). Moreover, the gene frequency of *IGF-1* c.258 A > G among pigs with different body size was excavated according to the whole-genome shotgun contigs (gws) and RefSeq genome databases. Results found that codon usage preference for this synonymous mutation was significant between the large and miniature pigs. At this synonymous mutation site, GCG encoding Ala is used in almost all large pigs, whereas allele A is more commonplace in miniature pigs (Figure 1D; Table 1). Additionally, *IGF-1* c.258 A > G not only showed conservative preference among different pig species with different body size but also indicated a distribution pattern related to animal body size among common mammal species (Table 2). It is worth noting that, for the conserved *IGF-1* gene, the synonymous codon has a high degree of preference and regularity and may likely have potential functional and genetic value under the long-term biological selection background.

ABEmax induced A-to-G base conversion in mice blastocysts

Base editing was conducted in mice embryos using microinjection of ABE-encoding mRNA and associated single-guide RNAs (sgRNAs). Two target sites from *IGF-1* were selected to identify the target sequences and perform the single base-editing process (Figure 2A). For the purpose of verifying the effectiveness and accuracy of the target sites, we evaluated base-editing efficiency in mice blastocysts. Notably, a targeted point mutation in IGF-1 was observed in 33.3% blastocysts (Table 3), and *IGF-1*-homozygous (*Ho*), *IGF-1*-heterozygous (*He*) and *IGF-1*-wild-type (*WT*) genotypes were detected by identifying the peaks of A and G in the DNA sequences (Figure 2D). Here, embryo identification demonstrated that the ABEmax system converted the adenines at positions 5−8 of protospacer to guanine effectively, and neither indels nor any other nucleotide changes were observed at the target sites, which is consistent with the result of Liu's group in mammalian cells.[20] Furthermore, no off-target mutations were detectable at potential off-target sites (POTs) in these loci of mutant blastocysts, and no indels or other mutations in the base window positions were detected. Overall, these results suggested that the ABEmax system is efficient and precise to introduce the conversion of the A-to-G base pair in mice blastocysts, and the sgRNAs we designed have certain targeting activity.

Base conversion at *IGF-1* c.258bp to generate editing mice

Due to the highly conserved coding sequence of *IGF-1* and the codon preference of Ala between pigs and mice (Figures 1B and 1C), subsequently, we transplanted mice embryos into surrogate mothers after microinjection and further explored the use of ABEmax to generate F0 generation mutant mice (Table 4). Here, a single A-to-G conversion is expected to yield a synonymous mutation *IGF-1* c.258 A > G in mice. The result showed that 32 pups were obtained, and 11 of 32 (34.4%) carried a desired Ho or He mutation at the target site (Table 5). Furthermore, no unexpected indels or other mutations in the base window positions were detected in F0-generation mice. However, only one case of proximal off target was detected and removed for the following reproduction. Moreover, Ho mutants without off-target effect in F0-generation mice were mated with WT mice to produce newborn F1-generation He mutant mice, illustrating that the target mutation could be stably transmitted to the offspring. Then hybridization was used to produce F2-generation base mutation mice with stable phenotype for further detection. According to the birth information of F2-generation mice, the synonymous mutation would not interfere with the reproductive performance of the base-editing mice (Table 6). Finally, we obtained a stable, inherited single-base mutation mouse model.

Off-target analysis in *IGF-1* c.258 A > G base mutation mice

To test whether off-target effects occurred in these genetically modified mice, we screened the mice genome and predicted five POTs for each sgRNA. The position of POT sequences, mismatch information, and PCR primers was listed in Table 3. Genomic DNA from the base-editing mice was amplified and identified using Sanger sequencing. The sequence results indicated that none of the sequencing reads exhibited mutation, suggesting that no obvious off-target mutations were detectable at POTs in mutant mice derived from the dual sgRNA system with cytoplasmic microinjection in pronuclear-stage embryos (Figure 3).

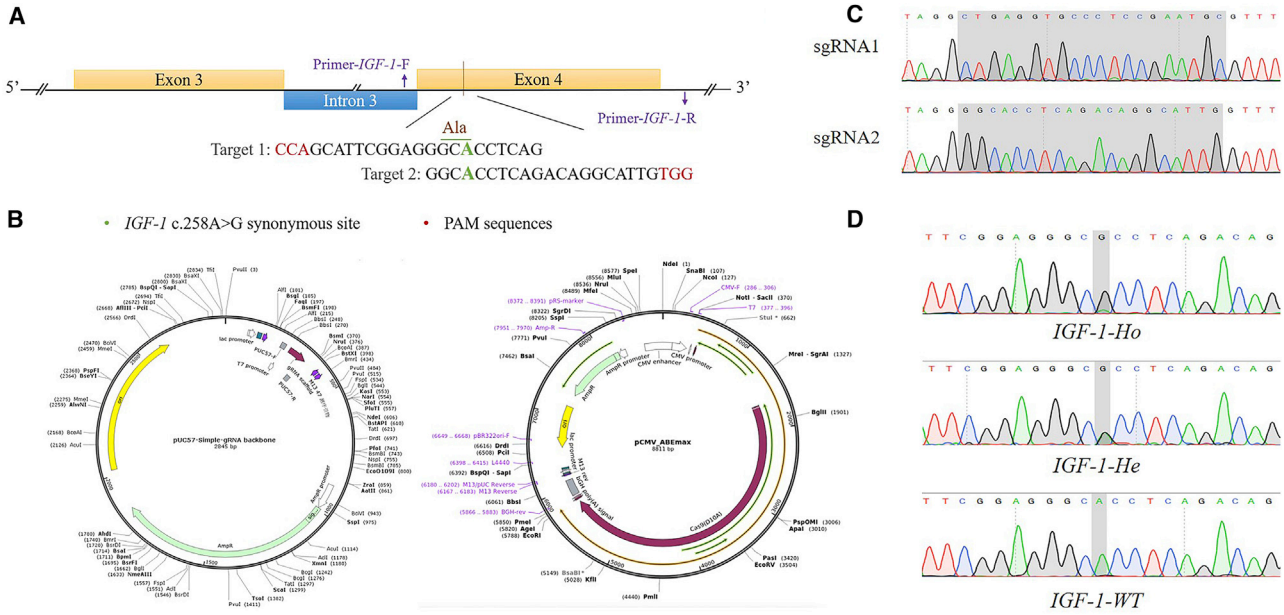

Figure 2. Construction of individual *IGF-1* c.258 A > G base-editing mice

(A) Schematic diagram of sgRNAs targeting the mus-*IGF-1* c.258 A > G synonymous site. Primer-*IGF-1*-F and primer-*IGF-1*-R were used to identify genotypes later, and the sequences were detailed in Table 2. Red bases: protospacer adjacent motif (PAM) regions. Green bases: *IGF-1* c.258 A > G synonymous mutation site. (B) The profiles of ABEmax-Cas9 and pUC57-sgRNA recombinant vectors. (C) The sequencing results of sgRNAs in pUC57-Mus-IGF-1-sgRNA recombinant vectors. (D) The sequence diagram of homozygous (Ho), heterozygous (He), and wild-type (WT) mice.

IGF-1 c.258 A > G synonymous mutation changes IGF-1 expression and secretion

It has been reported that the synonymous mutation *IGF-1* c.258 A > G may affect gene expression in cells. To further detect the influence and degree of the SNP in *IGF-1* at the animal level, gene-expression detecting and serological analysis were performed at different developmental stages in WT, He, and Ho mice. Studies have shown that breast milk contains IGF-1 for the growth and development of pups. In order to avoid the interference of IGF-1 in breast milk, we weaned mice at 3 weeks of age and monitored the expression and secretion of the *IGF-1* gene at 4 weeks of age (post-weaning period), 6 weeks of age (sexual maturity period), and 8 weeks of age (body maturity period).[24] Here, for male mice, the mRNA expression of Ho mutant mice was significantly downregulated at 6 weeks of age compared with He or WT mice (Figure 4A) ($p < 0.01$). A slight decline tendency can still be observed with 6-week-old male mice and 6-week-old female mice, respectively, even though the difference is not significant due to the individual variation (Figure 4A) ($p > 0.05$). Besides that, there were similar differences at 8 weeks of age (Figure 4A). In addition, as a secretory molecule protein, IGF-1 can be secreted into the serum and used throughout the body. It was found that the serum IGF-1 concentration of fast-growing pigs was significantly higher than that of slow-growing pigs in the same period.[25] For our base-editing mice, the serological analysis with enzyme-linked immunosorbent assay (ELISA) results demonstrated that the content of serum IGF-1 of Ho mutant mice was significantly downregulated at 8 weeks of age compared with He or WT mice (Figure 4C) ($p < 0.05$), which is consistent with our gene-expression results shown in Figure 4A. After that, the IGF-1 protein was detected by western blot (WB) and ELISA, and the results indicated that at 8 weeks of age, *IGF-1* expression in the liver of both male and female base-editing mice was significantly lower than that of WT mice (Figures S1 and 4D) ($p < 0.05$). Finally, we analyzed the total content of IGF-1 in liver tissues and serum. The results showed that IGF-1 protein was significantly decreased ($p < 0.01$). We hypothesized that *IGF-1* c.258 A > G had a greater effect on the protein translation rate, which also supported the conclusion of the relevant literature—that the functional mechanism of synonymous mutations is carried out in a translation-dependent manner.[23]

Table 3. Data statistics of A/G conversion efficiency in blastocysts using the ABEmax system

Recipients	sgRNA-Cas9 mRNA (ng/μL)	Embryos injected (N)	Blastocysts obtained (N)	No. of targeted mutation (N)	Mutant ratio (%)
1	50/100	10	9	2	33.33
2	50/100	13	9	2	

Table 4. Generation of targeted mutations in *IGF-1* c.258 A > G base-editing F0 mice

Recipients	Embryos transferred (% microinjected)	Pregnancy	No. of offspring	Male/female
1	11	yes	6	2/4
2	13	yes	8	4/4
3	10	yes	5	3/2
4	12	yes	6	2/4
5	12	yes	7	2/5

IGF-1 c.258 A > G synonymous mutation changes the mRNA expression of IGF-1-related genes

In order to reveal the effect network of the synonymous mutation, the expression of IGF-1-related genes in mice with different genotypes was further explored. Growth hormone (GH) is an important regulator of IGF-1 secretion and will produce negative feedback to the change of IGF-1 expression. Additionally, insulin like growth factor binding protein 3 (IGFBP3) is the most important IGF-1 binding protein in circulation. Thus, mRNA expression levels of these two IGF-1-related genes were analyzed. Results showed that the mRNA expression level of GH in the pituitary gland of Ho mutant mice was significantly increased (Figures 5A and 5B) ($p < 0.05$), whereas the expression of IGFBP3 in the liver had a tendency to increase, but no significant difference was found (Figures 5C and 5D) ($p > 0.05$).

Effects of *IGF-1* c.258 A > G synonymous mutation on body-shape formation and bone-tissue development in mice

Research has shown that the expression level of IGF-1 affects its biological function to varying degrees.[1,26] To further investigate whether and to what extent mice carrying different genotypes of IGF-1 have potential effects on growth and development, body-shape traits were tested for reference. To determine whether *IGF-1* c.258 A > G synonymous mutation affects body weight of ICR mice, weighing was administered every 3 days for 10 weeks, whereas the difference in daily food intake was not significant throughout the experiment. The body weight of the F2-generation mice increased steadily with small fluctuations until the mature stage and then trend rate of growth remained relatively stable. At the same time, the weight growth curves showed no difference in body weight of both male and female mice with different genotypes (Figure 6A) ($p > 0.05$). Similarly, tail length in mice at different developmental stages was evaluated, and the result showed no significant effect on tail-length formation with different genotypes (Figure 6B) ($p > 0.05$). Moreover, research has confirmed that IGF-1-overexpressing mice have larger organ weight and corresponding increased tissue volume in proportion to WT mice.[4] We further assessed the organ weight of 8-week-old mice, and the visceral index remained constant among the mice of WT, He, and Ho genotypes (Figure 6C) ($p > 0.05$).

As one of the most abundant growth factors in the bone matrix of adult mammals, IGF-1 is deemed to be closely related to the growth and development of skeleton, which is the basis of animal body size. Matilda H. C. Sheng et al.[27] confirmed that IGF-1 conditional knockout mice produced less IGF-1, which severely restricted the normal growth and development of bone.[27] Similarly, IGF-1 deficiency can affect the accumulation of sclerotin at almost all age stages, resulting in short stature.[28] To further explore whether this synonymous mutation affects the growth and development of bone, systemic X-ray was conducted in both male and female mice of 8 weeks old, and the result showed that the mutant mice had no skeletal dysplasia traits (Figure 6D) ($p > 0.05$). Alcian blue−alizarin red staining and hematoxylin and eosin (H&E) staining revealed bone epiphyses and femoral growth plates have no significant developmental abnormalities between the WT and Ho mice at 8 weeks of age (Figures 6E and 6F) ($p > 0.05$). With the combined body-shape traits of mice, these results may suggest that the effects of synonymous mutations of *IGF-1* are not sufficient to impact animal body size.

The synonymous codons of Ala in *IGF-1* affect gene expression and secretion

A certain decrease in gene-expression level was indeed detected, although the influence of *IGF-1* c.258 A > G synonymous mutations was not enough to change the development of mice body size and bone tissue. Therefore, the molecular mechanism of the influence of synonymous codons on gene expression was further explored.

The researchers proposed that, according to the ribosome-profiling assay, rare codons introduced by synonymous mutations may lead to the widespread presence of strong ribosomal pauses, which are restricted by the transfer RNA (tRNA) identity and abundance, followed by a decrease in protein translation rate and stability, thus leading to a decrease in gene expression.[10,29] For the purpose of exploring the influence of tRNA identity and abundance on gene expression, we designed individual reporters (mini-gene)[23] that contained coding sequences, in which all positions encoding by Ala possessed a single codon type. First, we replaced all Ala in IGF-1

Table 5. Summary of embryo development and A/G conversion efficiency in F0 mice using the ABEmax system

No. of zygotes	No. of 2-cell (%)[a]	No. of blastocysts (%)[a]	No. of offspring (%)[a]	No. of targeted mutation (%)[b]	No. of proximal off-targets (%)[b]	No. of homozygous target mutants (%)[b]	No. of heterozygous target mutants (%)[b]	No. of non-A to G (%)[b]
81	68 (83.9%)	58 (71.6%)	32 (47.1%)	11 (34.4%)	1 (3.1%)	3 (9.4%)	8 (25%)	21 (65.6%)

[a]Calculated from the number of zygotes.
[b]Calculated from the number of offspring.

Table 6. Birth information among F2 generation of *IGF-1* c.258 A > G base-editing mice

Recipients	Pregnancy	Mice obtained	Male/female	Mice with mutation	No. of homozygous (%)	No. of heterozygous (%)	No. of wild type (%)
1	yes	11	6/5	5	2 (18.2%)	3 (27.3%)	5 (45.5%)
2	yes	14	7/7	10	3 (21.5%)	7 (50%)	4 (28.6%)
3	yes	19	10/9	13	7 (36.8%)	6 (31.6%)	6 (31.6%)
4	yes	17	9/6	15	3 (17.6%)	12 (70.6%)	2 (11.8%)
5	yes	15	11/3	13	8 (53.3%)	5 (33.3%)	2 (13.3%)

with four synonymous codons, respectively, to expand the effect of each synonymous mutation. To determine whether the effect of a synonymous mutation is dependent on its sequence contexts,[30] a GCG/GCA-inverted IGF-1 plasmid was constructed. Besides, the IGF-1 genotype preferred by the large pigs (consistent with the IGF-1 genotype in PK-15 cells) was used as the IGF-1-WT group (Figure 7A). Then, the expression vectors that contained different synonymous codons were constructed and transfected into PK-15 cells. In order to avoid the interference of intracellular IGF-1, the reverse primer was designed on the coding sequence of the FLAG tag, and we detected that the amplified product used the primer and ensured the detection results are not interfered (Table 7). Due to the enrichment on a single codon in each mini-gene reporter, the mini-gene reporters provide a way to analyze the regulatory properties at a single codon resolution. Results showed a significantly lower expression amount in all mini-gene reporters than that of the WT group, and a similar trend in tRNA abundance and codon preference was observed (Figure 7C) ($p < 0.05$). Even though the number and type of Ala codons in the

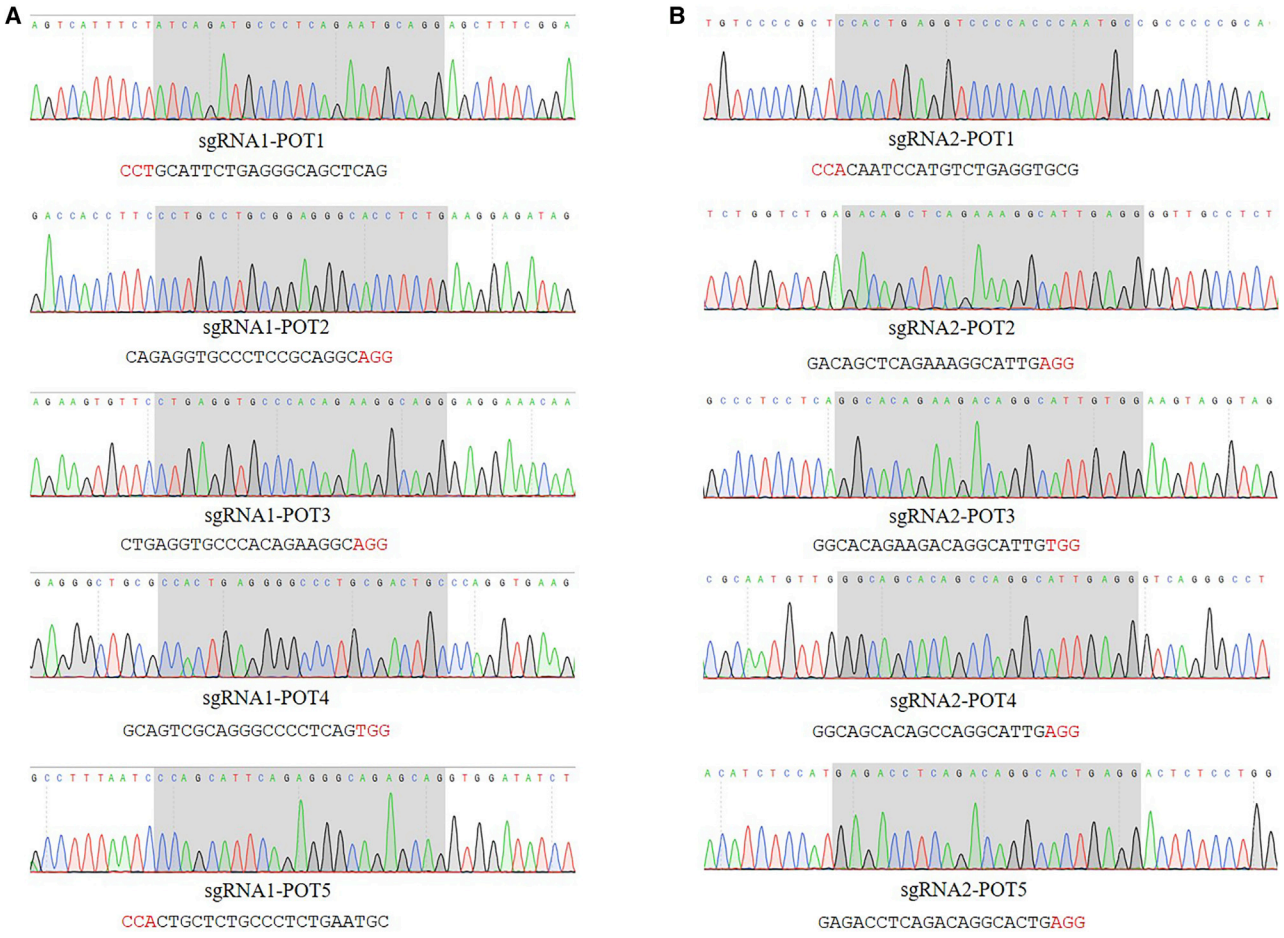

Figure 3. Potential off-target sequencing peak diagram in *IGF-1* c.258 A > G base-editing mice

(A) Identification of off-target sites for sgRNA1. (B) Identification of off-target sites for sgRNA2.

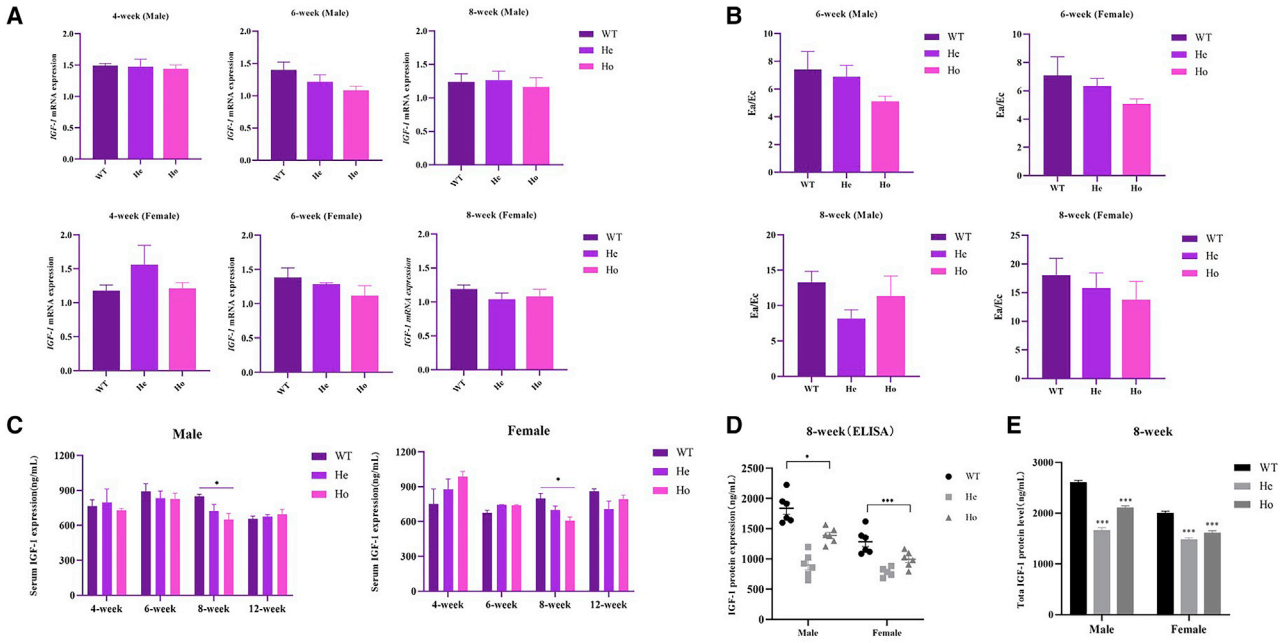

Figure 4. The effects of the synonymous mutation of *IGF-1* on related gene expression

(A and B) The mRNA levels of GH in *IGF-1* c.258 A > G base-editing mice. (C and D) The mRNA levels of IGFBP3 in *IGF-1* c.258 A > G base-editing mice.

invertion (IGF-1-INT) group were the same as the IGF-1-WT group, its mRNA expression level was still significantly lower than the IGF-1-WT group (Figure 7B) ($p < 0.01$). Then the protein expression levels were detected by WB with the anti-FLAG tag antibody 48 h after transfection, and the result showed that none of these groups had the higher IGF-1 protein expression level than that of the WT group ($p < 0.05$). The IGF-1 protein expression in the IGF-1-GCA group and the IGF-1-GCG group was significantly lower than that in the IGF-1-WT group ($p < 0.05$), whereas there was no significant difference between the two groups ($p > 0.05$). On the other hand, the IGF-1-GCC group and IGF-1-GCT group showed no significant difference compared with IGF-1-WT group (Figure 7C) ($p > 0.05$). These observations further validate the differences in optimality between synonymous codons for Ala.

The synonymous codons of Ala in *IGF-1* affect mRNA stability and the protein half-life

The replacement of the third synonymous site causes fluctuations in GC content and changes in the secondary structure and stability of mRNA.[31,32] Thus, we determined the mRNA stability and found the inhibition level of *IGF-1* gene transcription was significantly different between different groups by blocking the mRNA transcription using actinomycin D (ActD) (Figure 8A) ($p < 0.05$). The mRNA stability of almost all mini-gene reporters was higher than the IGF-1-WT group to some degree, except that the IGF-1-GCT group and IGF-1-WT group were consistent, in which the stability of the IGF-1-GCA group was significantly higher than the IGF-1-WT group at any time point (Figures 8B and 8E) ($p < 0.05$). Compared with IGF-1-WT group, IGF-1-GCG, IGF-1-GCC, and IGF-1-INT groups were less susceptible to transcriptional suppression at 1 h (Figures 8C, 8D, and 8F) ($p < 0.01$), and the IGF-1-GCC group was still significantly stable at 2 h ($p < 0.001$). Given the above, IGF-1 mRNA half-life is the longest in IGF-1-GCC group and IGF-1-GCA group, followed by the IGF-1-GCG group and IGF-1-INT group, whereas the IGF-1-WT group and IGF-1-GCT group have the lowest IGF-1 mRNA stability (Figures 8G and 8H) ($p < 0.05$). Furthermore, we also detected whether the IGF-1-GCG group was decayed faster than the IGF-1-GCA group (Figures 8B and 8C) ($p < 0.05$), which is consistent with previous research.[8] Similarly, cycloheximide (CHX) was used to block protein synthesis. Over time, as the synthesis of the nascent chain was inhibited, the synthesized IGF-1 is gradually utilized or degraded. In this way, the protein stability of different genotypes of *IGF-1* was evaluated by examining the IGF-1 content at desired time points. The results declared that IGF-1-GCG and IGF-1-GCC groups were reduced slower than the IGF-1-WT group (Figures 8H–8M) ($p < 0.05$).

The synonymous codons of Ala in *IGF-1* affect mRNA secondary structure and pre-mRNA splicing

Studies have shown that the synonymous rare codon, once thought to have only negative impacts on the speed and accuracy of translation, has strong heterogeneity.[10,23] Only one synonymous mutation producing Ala (c.258 A > G) was screened from the coding regions of *IGF-1* in different body-size pigs, and it is located in the pivotal coding position of the receptor-binding domain. To elucidate the potential impact of the synonymous mutation, the mRNA secondary structure and minimum free energy were predicted using the Mfold web server. Results showed that the mRNA secondary structures of the various

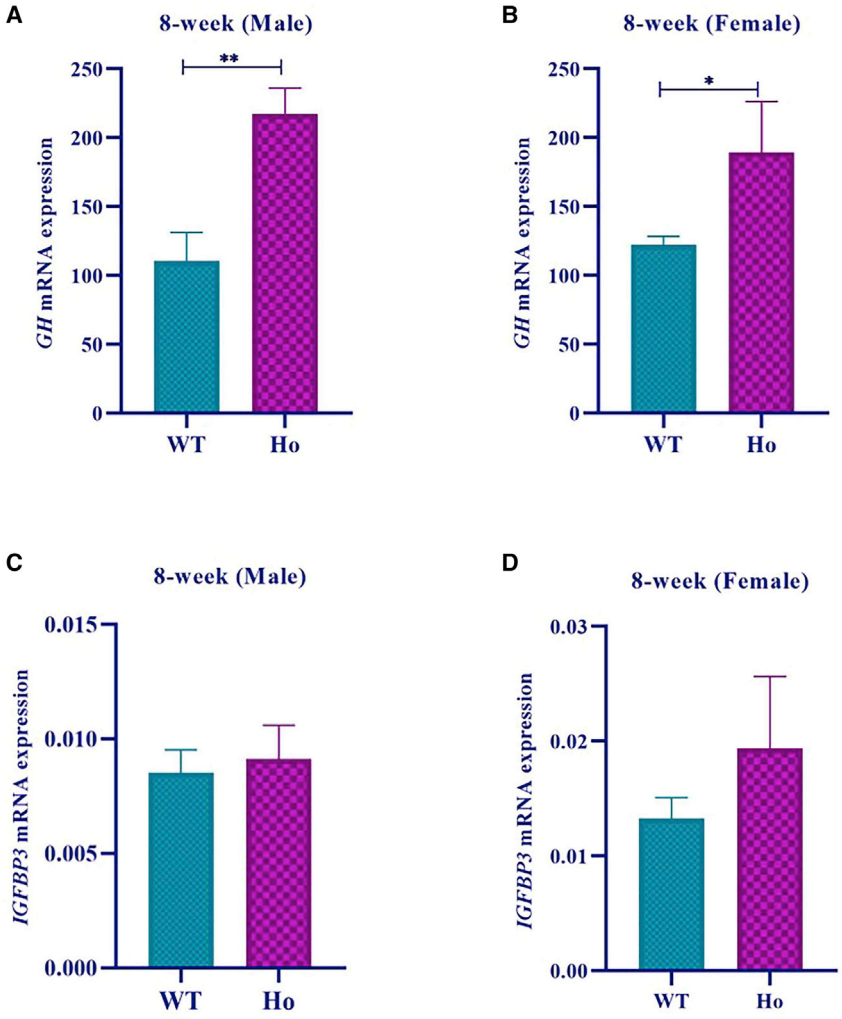

Figure 5. Phenotypic analysis of *IGF-1* c.258 A > G base-editing mice

(A) Broken lines of body weight of mice with different genotypes. (B) Statistics of tail length of mice with different genotypes at different developmental stages (4 weeks old, 6 weeks old, and 8 weeks old). (C) Viscera index of 8-week-old mice with different genotypes. (D) The bone development of mice with different genotypes measured by X-ray: (1) WT-female, (2) WT-male, (3) He-female, (4) He-male, (5) Ho-female, and (6) Ho-male.

coding sequences of IGF-1 mature peptides were changed due to the synonymous mutations (Figures 9A–9F). The minimum free energy has a 6.43 kcal or 0.1 kcal increase when the IGF-1-WT (Figure 9F) changed to IGF-1-GCC (Figure 9C) or IGF-1-INT (Figure 9E), whereas the minimum free energy of IGF-1-GCA (Figure 9A), IGF-1-GCG (Figure 9B), and IGF-1-GCT (Figure 9D) have declined by 7.91 kcal, 1.26 kcal, and 5.47 kcal, respectively, compared with IGF-1-WT (Figure 9F).

On the other hand, in this study, after observing a slight downregulation of gene expression, we further investigated whether the proportion of the variable splice isomers of IGF-1 changed. Ea and Ec peptides were detected by qRT-PCR from 6- and 8-week-old mice due to E peptides, which have also been shown to be related to bone development,[33,34] and the results showed that Ea/Ec in both male and female Ho mice were slightly lower than that of WT mice (Figures 4B and S2). Additionally, previous studies have demonstrated that synonymous codon replacement of exonic splicing enhancers (ESEs) or exonic splicing silencers (ESSs) leads to differences in the relative abundance of mRNA or protein structure and causes human diseases, including familial growth hormone deficiency.[35–37] Studies still have indicated that the secondary structure of mRNA cannot only affect the mRNA stability but also affects the binding of RNA-binding protein (RBP), thus affecting the accuracy and normal conduct of splicing.[38] ESEfinder3.0 ESE finder (http://rulai.cshl.Edu/tools/ESE/) was used to identify putative ESEs responsive to the SR proteins and to predict whether exonic mutations disrupt such elements.[39] Results suggested that this synonymous mutation site is located in a motif (5′-CG/ACCACAG-3′; score = 2.92762/2.566766 > 2.383) of potential binding to SR protein SC35, even though the binding capacity was only slightly changed. Meanwhile, when the allele G was mutated to A, a potential SF2/ASF (a prototypical serine- and arginine-rich protein, with important roles in splicing and other aspects of mRNA metabolism) ESE motif (5′-GGCACCA-3′; score = 1.986822 > 1.956) and a potential SC35 ESE motif (5′-CGCCACAG-3′; score = 2.92762 > 2.383) were predicted, which may be one type of the mechanism that leads to the imbalance of the variable spliceosome of IGF-1 and needs further investigation (Table 8).

Effects of eukaryotic synonymous mutations of Ala in *IGF-1* on protein biosynthesis

The structure of mRNA not only influences mRNA synthesis, stability, and splicing events but also has been found to influence almost every step of protein biosynthesis.[40] More importantly, in the quantification process of the impact of a single synonymous mutation, it was found that the impact of a single synonymous mutation on mRNA level depends on the regulation of proximal sequence codon preference and secondary structure, and the replacement of the synonymous codon has multiple effects, and the mRNA level difference caused by it is also reflected in the protein level.[30] Furthermore, examples of structural differences in mRNA caused by synonymous mutations have been shown to alter the rate of protein synthesis.[41,42] Thus, by adding the same amount of mRNA, an *in vitro* translation experiment was used to determine the initiation

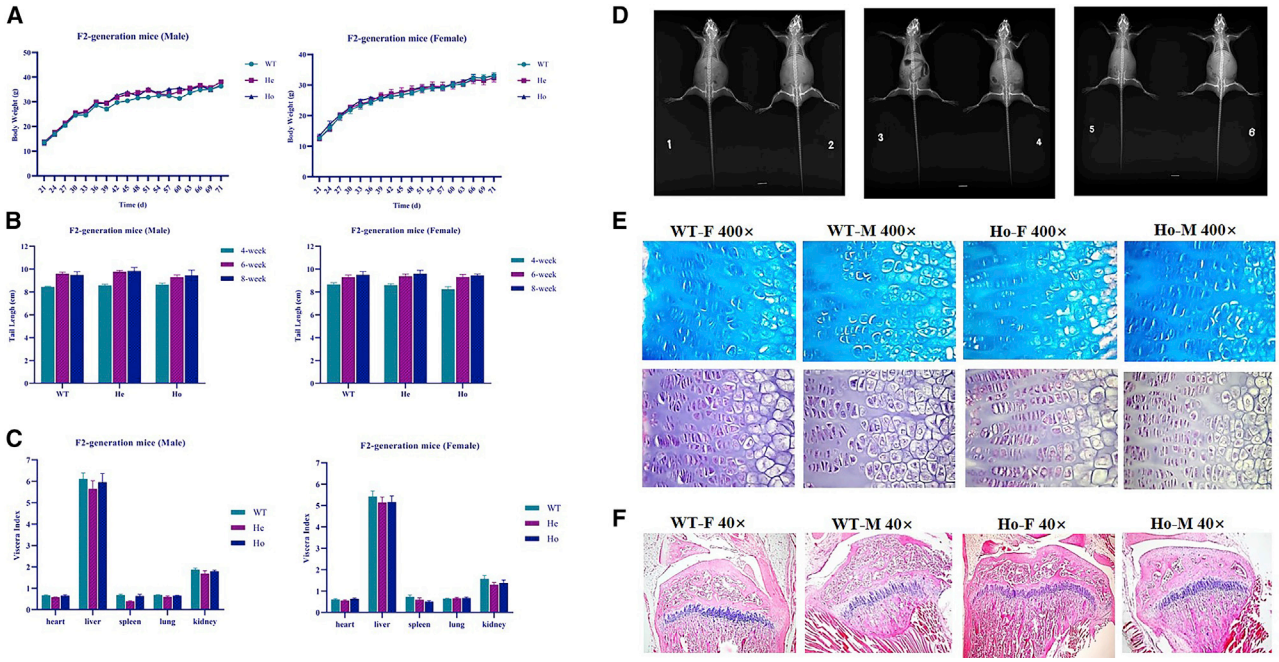

Figure 6. Body-type phenotyping of F2-generation mice with different genotypes

(A) Broken lines of body weight of mice with different genotypes. (B) Statistics of tail length of mice with different genotypes at different developmental stages (4 weeks old, 6 weeks old, and 8 weeks old). (C) Viscera index of 8-week-old mice with different genotypes. (D) The bone development of mice with different genotypes measured by X-ray: (1) WT-female, (2) WT-male, (3) He-female, (4) He-male, (5) Ho-female, and (6) Ho-male. (E) Alcian blue–alizarin red stain of mice bone epiphyses with different genotypes at 8 weeks of age. (F) H&E staining of femoral growth plates of mice with different genotypes at 8 weeks of age.

efficiency of translation synthesis. Quantitative analysis of translation products by ELISA suggested that different synonymous codons can indeed affect the efficiency of translation initiation in the case of consistent translation efficiency, and the IGF-1-WT group had the highest initial translation efficiency. The protein synthesis efficiency of the IGF-1-GCA, IGF-1-GCC, and IGF-1-GCT groups was significantly lower than that of the IGF-1-WT group (Figure 9G) ($p < 0.05$). The mRNA stability of the IGF-1-GCA and IGF-1-GCC groups was relatively high, and the initiation rate of protein synthesis was lower than others ($p < 0.05$). Therefore, except the IGF-1-GCT group, it basically conforms to the hypothesis that high stability of mRNA will reduce the initial efficiency of protein synthesis; however, it also indirectly indicates that the protein biosynthesis process may be restricted by other factors (Figures 8A and 9G).

The synonymous codons of Ala affect IGF-1 biological function

Autocrine/paracrine IGF-1 is also an important determinant of growth after birth.[43] At the same time, synonymous mutations have been reported to affect protein-specific function activities.[44] Here, we observe that Ala codon influences can affect the structure and expression of IGF-1 protein to some extent. Studies have shown that in the neuroendocrine growth axis system, IGF-1 regulated key pathways of cell proliferation and further affected the growth and development of animals.[1,2] Therefore, we examined whether specific synonymous codons would affect the proliferation function of IGF-1. Results demonstrated proliferation viability of the PK-15 cells in the IGF-1-GCG group, IGF-1-GCC group, and IGF-1-INT group was significantly weaker during 0–48 h compared with the IGF-1-WT group (Figures 10A, 10C, 10D, and 10F) ($p < 0.05$). To sum up, consistent with the gene-expression results, the proliferation ability of the IGF-1-WT group was almost the best, which suggests the mRNA stability and limiting translation initiation mechanism may have some offsetting effects. Interestingly, cells in IGF-1-GCC group showed a slightly higher proliferation efficiency within 48−96 h compared with the IGF-1-WT group ($p < 0.01$), which may indicate that codon preference and tRNA abundance are the key sources. The growth trend of cells in the IGF-1-GCA group was similar to that of the IGF-1-WT group; however, the result did not amplify the inhibitory effect of cell proliferation as expected (Figure 10B). Among different possible mechanisms, even if GCA is not a common codon, mRNA stability was thought to play a potentially decisive role. Moreover, the introduction of a rare codon may change the protein conformation and the binding efficiency of its receptor IGF-1R, which may not be a negative effect,[45] and data indicated that cells of the IGF-1-INT group exhibited reduced growth potential compared to that of the IGF-1-WT group (Figure 10F) ($p < 0.05$), which suggests the location of the synonymous codon and sequence context affects the mRNA stability and encoded protein function, although the differences in protein expression and protein stability between the two groups are not significant (Figures 8, 9, and 10F) ($p > 0.05$).

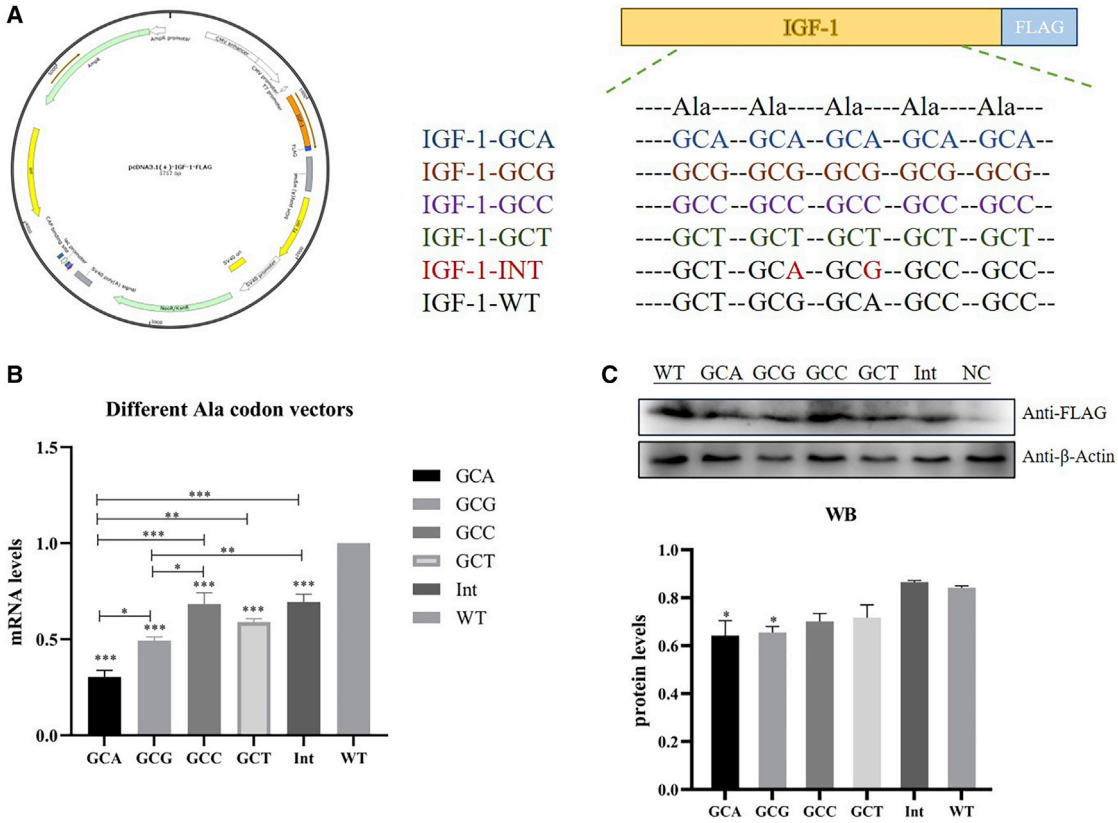

Figure 7. The synonymous mutation of *IGF-1* affects gene expression in transfected PK-15 cells

(A) Construction diagram of expression vectors carrying different synonymous codons. (B) mRNA levels of IGF-1 with different synonymous codons at 36 h after transfection. (C) Protein levels of IG-1 with different synonymous codons at 48 h after transfection.

DISCUSSION

IGF-1 as a growth factor with the regulating function of animal growth is produced mainly by the liver and acts on almost all tissues or organs.[46] Research has indicated that IGF-1 deficiency can induce short stature.[47] Moreover, in the previous study, a significant expression difference was detected in the *IGF-1* gene between the different body-size pigs in liver and muscle tissues. Only one synonymous mutation c.258 A > G was screened in the *IGF-1* gene between large white pigs (large pigs) and Bama Xiang pigs (miniature pigs), which had the opposite growth rate and body size.[8]

In general, common codons are thought to be more preferred in the evolution. However, negative selection does not eliminate rare codons, and some synonymous codons continue to survive as a result of mutation drift, even though rare codons tend to result in low translation speed and fidelity.[48,49] These findings have also validated the hypothesis that rare codons are likely to be positively selected in some specific sites, which may have functional effects.[50] Due to the low translation accuracy caused by rare codons, researchers speculated that they are in strong negative selection for significant amino acid regions, and rare codon clusters may be confined to unusual or rarely expressed genes, with weak selection for translation efficiency.[51,52] Nevertheless, many highly expressed genes, including genes for ribosomal proteins, were also found to contain rare codon clusters.[53] The uncertainty of these conclusions underscores the importance of experimental data in verifying the function of codon usage. Nathan B. Sutter et al.[54] demonstrated that a single *IGF-1* SNP haplotype is common to all small breeds and nearly absent from giant breeds, suggesting that the same causal sequence variant is a major contributor to body size in dogs. These results provide a precedent for our study aimed at identifying the genetic basis for complex growth traits with miniature pigs that may have experienced strong natural selection. Similar studies indicated that similar synonymous mutations persist and remain stable in certain populations over long periods of reproduction and domestication.[55] Therefore, the selection mechanism of a single synonymous mutation has strong heterogeneity characteristics. Here, we suggest that the previously screened *IGF-1* synonymous mutation has a potential selection effect and may be significantly related to the formation mechanism of miniature pigs.

At present, the mechanism of a single synonymous mutation in a specific gene subjected to genetic selection has not been studied. Our study confirmed that the synonymous mutation in *IGF-1* resulted

Table 7. IGF-1-FLAG qRT-PCR primer information

Primers	Sequence (5′ → 3′)	PCR product (bp)	Extension time (s)	Annealing temperature (°C)
IGF-1-FLAG-F	CACATCACATCCTCTTCGCATC	309189	2520	58.558.5
IGF-1-FLAG-R	TCACTTATCGTCGTCATCCTTGTAA			
β-actin-F	TGTGCAGGGTATTCATGTGTCCGA			
β-actin-R	CAAGGCAAGTTAACAACCCACGGT			

in a certain amount of decline in gene expression and secretion levels but not enough to induce significant phenotypic changes. For the unstable results in animal experiments, individual differences were taken into account first, and the influence of a SNP may be disturbed by multiple factors such as the feedback and neutralization of related biological pathways. Results showed that at the protein expression level, IGF-1 was slightly downregulated in both male and female mice at 6 weeks and 8 weeks of age. The possible reason was that the growth status of 4-week-old mice may be affected and limited by breastfeeding, and the number of offspring and was less regulated by their own secretion of IGF-1. Additionally, the sexual maturation process may be regulated by a variety of hormonal interactions. As a downstream molecule of the neuroendocrine axis, the role of IGF-1 in the complex factor network needs to be further explored. After 12 weeks of age, the mice were no longer in the growth and development stage with a gradual decrease in IGF-1 demand and then maintain steady state, so it may be difficult to observe the effect of SNP.

In addition, the effect of the synonymous mutation on different stages of animal growth and development is different. The synthesis and utilization of IGF-1 reached a peak at 8 weeks of age when the mice were mature. We hypothesized that although synonymous mutations had little effect on animal body-type traits, they might be more significantly restricted by codon colligation when IGF-1 has a high utilization rate, so IGF-1 expression and secretion were somewhat different at 8 weeks of age.[56] At other stages of growth, IGF-1 demand is relatively low, and then changes in gene expression are almost undetectable. In other tissues and organs, IGF-1 production and secretion are much less than in the liver and thus may be more strongly influenced by codon preference. Moreover, detectable circulating IGF-1 levels may not be reflective of the concentration of IGF-1 at the tissue level, where it may be locally produced and exert autocrine and paracrine function. Thus, it is possible that declining circulating levels of IGF-1 may induce greater local tissue production of IGF-1 as a result of loosened negative feedback that may be exerted by circulating IGF-1. Thus, different organ systems with various IGF-1 requirements may maintain optimal function despite a systemic decline in circulating IGF-1.[57] It followed that the role of synonymous mutation is highly heterogeneous, and its mode and degree of action still need more basic studies to confirm.

However, base editing did break the original codon usage rule of IGF-1 in mice. Even if the introduction of such mutations did not cause obvious changes in the body size of animals, and the effect on the

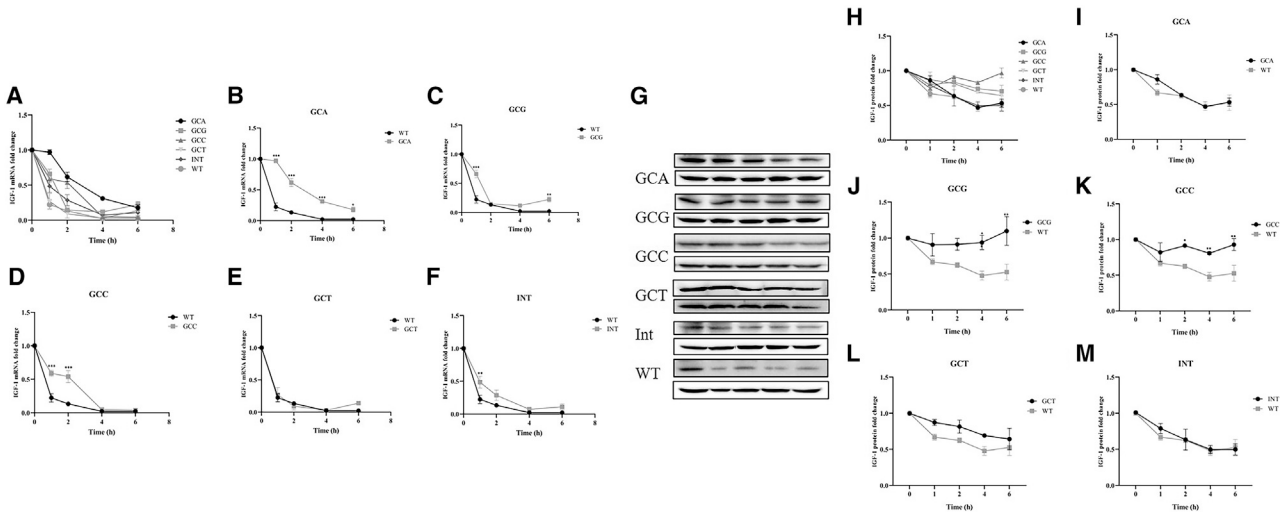

Figure 8. The synonymous mutation of *IGF-1* affects gene stability

(A) The effect of different codons on IGF-1 mRNA stability. (B–F) The mRNA stability differences between the WT group and IGF-1-GCA, IGF-1-GCG, IGF-1-GCC, IGF-1-GCT, and IGF-1-INT groups, respectively. (G) The effect of different codons on IGF-1 protein stability according to WB. (H) The effect of different codons on IGF-1 protein stability. (I–M) Protein stability differences analyzed by gray scale between the IGF-1-WT group and IGF-1-GCA, IGF-1-GCG, IGF-1-GCC, IGF-1-GCT, and IGF-1-INT groups, respectively.

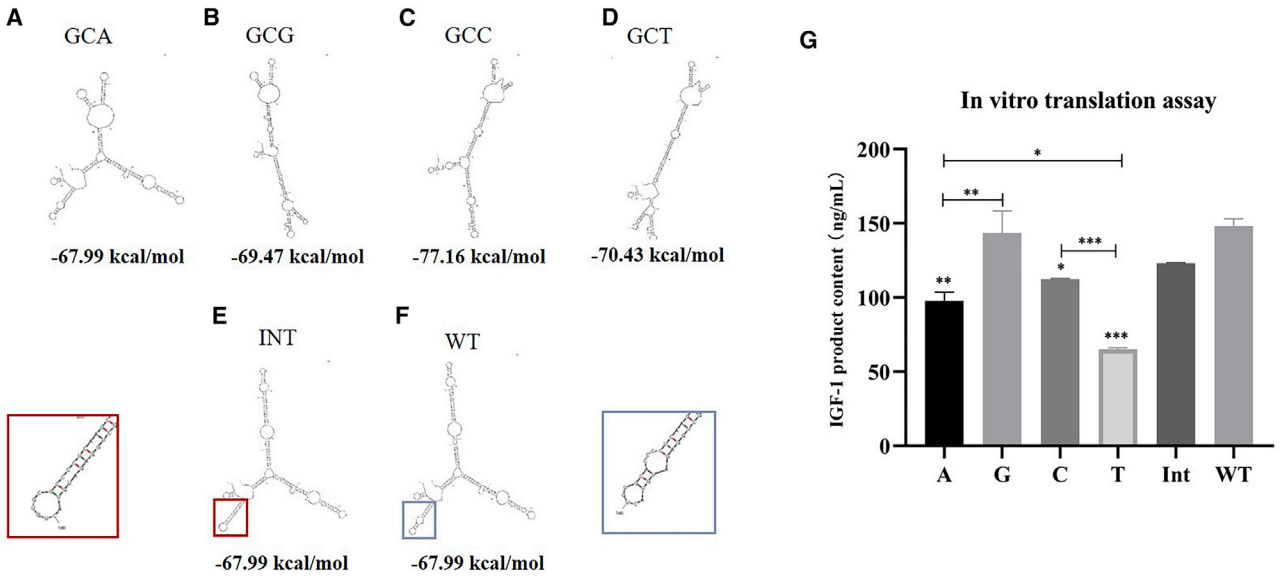

Figure 9. The synonymous mutation affects IGF-1 mRNA secondary structure and protein translation initiation

(A–F) IGF-1 mRNA secondary structure prediction with different codons. (G) The effect of different codons on protein translation initiation.

growth and development function of mice was not significant, we still believe that the slight variation of gene expression has the value of in-depth study. In other words, why does substitution of the synonymous codon change the amount of gene expression?

Research supports the premise that codons encode more information than merely amino acids and demonstrated that synonymous polymorphisms can affect mRNA splicing, stability, and structure. Additionally, changes give insight into the role of translation in protein biosynthesis, including protein expression, translation initiation, protein-folding efficiency, and the coordinated regulation of functionally related genes.[29,37,45,58,59] It is worth mentioning that the use of the codon is positively correlated with tRNA content.[60,61] Rare codons usually interact weakly with lower cognate tRNA levels or with the wobble position of the same tRNA,[62–64] and experimental data support the hypothesis that rare codons are usually translated more slowly than common codons.[10,65–67] These shreds of evidence indicate that a synonymous mutation not only induces a change of one base but also changes the codon utilization of the coding amino acids and thus has potential regulatory effects on the translation rate and even the structure and function of proteins.

Taken together above, we further confirmed the effect of synonymous codon preference on *IGF-1* gene expression in PK-15 cells and explored whether the tRNA abundance is a key factor affecting the mRNA synthesis rate. According to the codon composition of Ala encoding the IGF-1 sequence of large pig breeds (considered to be the optimal codon composition) with high expression efficiency, it was found there was one GCG, one GCA, one GCT, and two GCC, which basically accord with the rule of codon usage frequency. However, the synonymous mutation we studied made one of the GCA encoding Ala replaced by GCG, which reduced the mRNA expression. Combined with the theory of tRNA abundance, the results were as speculated, although the mRNA and protein expression levels of both IGF-1-GCA and IGF-1-GCG were lower than that of the IGF-1-WT type, which proved that the low tRNA abundance of GCA and GCG may lead to *in vivo* competitive mechanisms, and the use of a large number of the same rare codon would inevitably restrict mRNA expression. At the same time, we investigated whether high frequency codons in the sequence would have higher gene expression than the IGF-1-WT group. However, the results showed that the expression level of the IGF-1-GCC group and IGF-1-GCT groups was relatively lower than the IGF-1-WT group. They also suggest that the widespread use of rare codons plays an important role in gene expression. They are not abundant but indispensable. Rare codons may relieve the pressure of commonly used codons and thus improve mRNA expression. Therefore, we infer that there is no best codon whether the frequency of codon usage is high or low. Correspondingly, the codon collocation should be examined more.

More importantly, the effects of the individual synonymous mutation of IGF-1 on expression level were quantified, and we found it depends on codon usage bias and mRNA secondary structure, both in proximal sequence contexts.[30] Results showed that the IGF-1-WT group was still the more dominant genotype after codon position was switched, indicating that in addition to optimizing the frequency of codon use, the codon position was also of important significance.

In recent years, the advanced research of mRNA stability indicated the close relationship between translation dynamics and the mRNA structure, which was linked to the theory of codon bias.[30,65,68] Therefore, we investigated the potential effects of different

Table 8. Potential exon splicing enhancer prediction at the synonymous mutation site of *IGF-1*

SR protein	Genotype (258 bp)	Position (bp)	Motif (5′ → 3′)	Score (threshold)
SF2/ASF	A	254	–	–
		255	GGCACCA	1.986822 (1.956)
		257	–	–
	G	254	–	–
		255	–	–
		257	–	–
SC35	A	254	CGCCACAG	2.577333 (2.383)
		255	–	–
		257	CACCACAG	2.566766 (2.383)
	G	254	–	–
		255	–	–
		257	CGCCACAG	2.92762 (2.383)

synonymous codons on mRNA secondary structure, mRNA stability, translation initiation process, and protein half-life. mRNA structure provides an additional mechanism to regulate translation initiation efficiency and translation extension speed, and particular mRNA structures can cause translational pauses.[69,70] Then, subtle changes of mRNA secondary structure resulting from the introduction of synonymous codon changes were illustrated. In terms of mRNA and protein stability, the IGF-1-WT group was considered to have the lowest stability, although promoting gene expression. The IGF-1-INT group was similar but not as extreme as the IGF-1WT group. Conversely, the IGF-1-GCA group had the most stable mRNA content and showed a relatively long protein half-life, whereas the upregulated protein levels in IGF-1-GCG and IGF-1-GCC groups in the late detection period may be attributed to higher gene-expression levels, superior translation initiation efficiency, and stronger cell proliferation ability. Furthermore, results illustrated that codon preference also plays a role in the regulation of gene function to a certain extent, and the preferred codons in large pigs are more conducive to cell proliferation and survival. Surprisingly, there was no significant inhibition of cell proliferation in the IGF-1-GCA group. These results implied that additional selective pressures govern the use of rare codons and specifically that local pauses in translation can be beneficial for protein biogenesis.[53]

It can be seen that the results of these mechanisms are not as regular as we expected, but there are significant differences among different synonymous codons. Moreover, we realized the substitution of multiple synonymous codons would not necessarily present a superposition effect and may be affected by the fluctuation of sequence context to some extent. We suggested that the work degree of diverse influencing factors may not be nearly the same. Generally speaking, the various mechanisms tend to balance dynamically, rather than amplify the differences.

Of course, there are some limitations to our results at the animal level. We examined only liver tissues that primarily secrete IGF-1. In addition, due to individual differences, we did not observe significant differences in IGF-1 mRNA levels but found differences in protein levels, which may be due to the fact that IGF-1-producing livers are more sensitive to the abundance and utilization of tRNA. Thus, tRNA collocation may be the major influencing factor. If it is in other organs of the body, it may be affected by codon usage preference to a greater extent.

On the other hand, some studies have linked longevity to growth. Ozanne and Hales[71] investigated that limiting growth during the suckling period not only increased longevity but also protected against the life-shortening effect of an obesity-inducing diet later on. By contrast, lifespan was considerably shortened if the postpartum growth period was accelerated to make up for lost growth in utero[71]. In many species, smaller individuals outlive larger ones, and studies of various human populations have shown a similar relationship.[72] Meanwhile, different research illustrated that the changes in either IGF-1 levels or the evolutionally conserved downstream signaling cascade among diverse species play a critical role in the regulation of aging and longevity,[73] and the genetic variability at the IGF-1 response genes was demonstrated to involve human longevity.[74] By far, the two most widely used models of GH/IGF-1 deficiency are the Snell and Ames dwarf mice, which demonstrated increased longevity relative to WT controls. The development of pituitary cells in these mutant mice was inhibited to the extent that production of the related hormones may be absent. Due to the complexity of the endocrine system, many cascade effects caused by excessive deficiency of growth-related hormones in model mice cannot be accurately estimated. It is still necessary to further test the hypothesis that GH/IGF-1 deficiency itself leads to prolonged lifespan indeed. Christy S. Carter et al.[75] suggested that in order to avoid

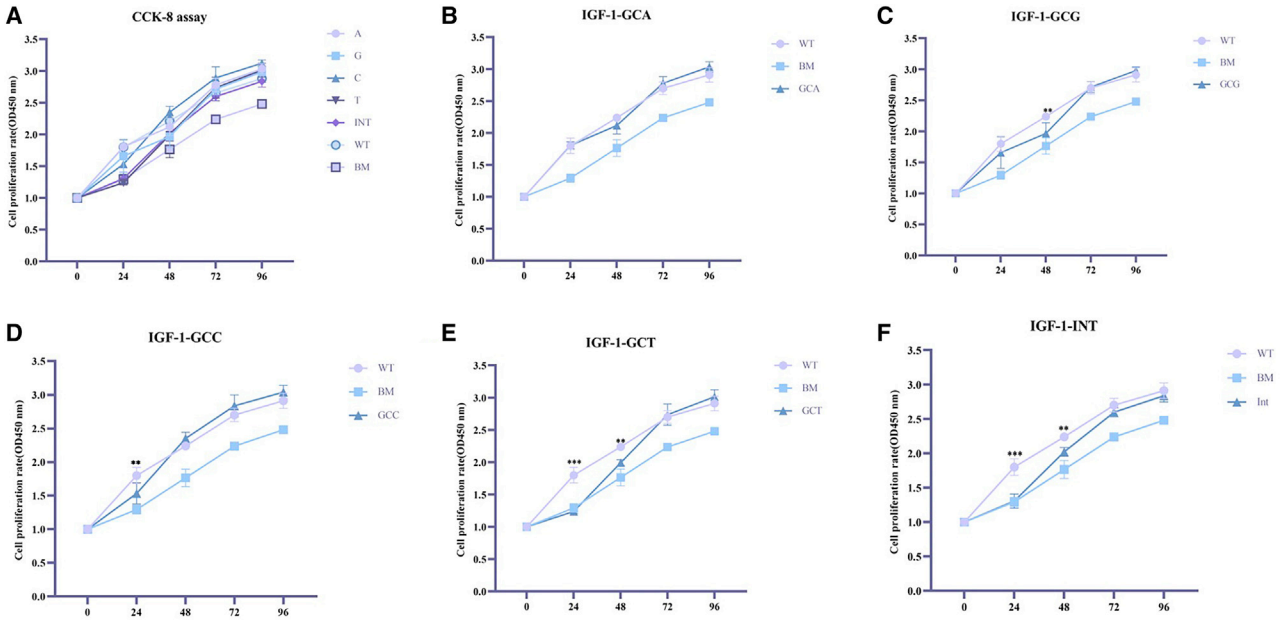

Figure 10. The synonymous mutation affects the function of cell proliferation

(A) Proliferation curves of groups with different genotypes. (B–F) The cell proliferation differences between the IGF-1-WT group and IGF-1-GCA, IGF-1-GCG, IGF-1-GCC, IGF-1-GCT, and IGF-1-INT groups, respectively.

the secondary and developmental effects, models of GH/IGF-1 deficiency should meet the following criteria: growth hormone must be present during critical developmental periods to ensure adequate organizational development, and the GH or IGF-1 deficiency must be limited to avoid secondary endocrine effects.[75] Nevertheless, the models are difficult to implement at present. Combined with the results of this study, our base-editing mice model may likely be modified and satisfy the reasonable IGF-1 deficiency model for investigating longevity.

Interestingly, we observed that the IGF-1 synonymous mutation can reduce expression at a relatively mild degree, which may be another application of synonymous mutation in the biomedical field. Compared to missense mutation, using the codon preference to reduce the protein content may be more valuable and safer. Without significant decrease in gene expression or protein structural disorder can avoid complex and inestimable functional changes. In addition, with the continuous innovation of base-editing methods, various types of point mutations and even accurate localization can be achieved. Therefore, on the premise of a clear study of a particular synonymous mutation, controlling the deletion degree of gene expression is to be expected. Of course, further studies are still needed to be explored.

In conclusion, the influence of a single synonymous mutation of IGF-1 in mice was validated the first time, and it can alter the gene expression at both transcription and translation levels, which may contribute to the formation of miniature pigs. Even though much remains still unknown about the molecular mechanisms connecting synonymous codon usage to efficient protein biogenesis and proper cell function, the translation dynamic effect induced by tRNA identity and mRNA structure was further observed through experimental data in our study. In addition, we indicated that a delicate dynamic balance among the different mechanisms constrained by synonymous codons enables the minimal functional changes due to codon substitution. Compared with the codon-optimization strategy, which has been widely used in industrial production to improve expression efficiency, the synonymous codon can also be used intentionally in various biological studies of subtle expression reduction.

MATERIALS AND METHODS

Animals and ethical statement

6-week-old ICR mice were obtained from Liaoning Changsheng Biotechnology and used as embryo donors and foster strains. All of the study protocols were approved by the Laboratory Animal Center of Jilin University (SYXK [Ji] 2016-0001) and conducted according to experimental practices and standards approved by the Animal Welfare and Research Ethics Committee guidelines on the ethical use of animals at Jilin University. Mice were raised and reproduced in the specific pathogen-free (SPF) animal facility and allowed to acclimate for 1 week before the conduction of experiments with free access to food and water. The light and dark cycles were 12 h/12 h, and the temperature was kept constant at 22°C.

Vector construction and sgRNA preparation

Considering the conservative properties of IGF-1 mature peptide, gene homology analysis was performed among the different mammal species and further construct a mouse model of *IGF-1* c.258 A > G synonymous mutation. According to the editing window, two pairs of sgRNAs

Table 9. Designing oligonucleotides for pUC57-Mus-IGF-1-gRNA vectors

Vectors	*Mus-IGF-1-sgRNA1* (5′→3′)	*Mus-IGF-1-sgRNA2* (5′→3′)
PAM sequence	TGG	TGG
Oligo(dT) primer-F	TAGGCTGAGGTG CCCTCCGAATGC	TAGGGGCACCTCA GACAGGCATTG
Oligo(dT) primer-R	AAACGCATTCGG AGGGCACCTCAG	AAACCAATGCCTGT CTGAGGTGCC

F, forward; R, reverse.

around the synonymous mutation homologous site were designed by the online prediction tool (https://zlab.bio/guide-design-resources). For construction of sgRNA expression vectors, the pUC57-Simple plasmid (Addgene; #51306) was linearized with *Bbs*I, and sgRNA-oligos were synthesized, annealed, and connected to the *Bbs*I site of the pUC57-sgRNA expression vectors with the T7 promoter. A pair of complementary oligonucleotides encoding the 20-nt guide sequences were annealed at 95°C for 5 min and ramped down to 25°C to generate the dsDNA fragment, which was then cloned into the *Bbs*I-digested gRNA expression vector. PCR products for *in vitro* transcription of gRNAs were amplified using T7-F: (5′-GAAATTAATACGACTC ACTATA-3′) and T7-R: (5′-AAAAAAAGCACCGACTCGGTGCC AC-3′) primers. Then, sgRNAs were amplified and transcribed *in vitro* using the MAXIscript T7 kit (Ambion) and purified with the miRNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. The concentration and quality of synthesized mRNAs were determined by the Nanodrop ND-2000 spectrophotometer (Thermo Scientific) and agarose gel electrophoresis, respectively. The pCMV-ABEmax plasmid (Addgene; #112095) was linearized with *Not*I, and mRNA was synthesized using the *in vitro* RNA transcription kit (HiScribe T7 ARCA mRNA kit; NEB). pCMV-ABEmax and pUC57 plasmids used in this study were gifted from Prof. Zhanjun Li, and sgRNA-oligo sequences were shown in Table 9.

IGF-1 expression vectors with different synonymous codons were constructed as follows. To explore the roles for synonymous codons, IGF-1 coding regions with different Ala coding codons (IGF-1-GCA, IGF-1-GCG, IGF-1-GCC, IGF-1-GCT, and IGF-1-INT) were synthesized by Sangon Biotech (Shanghai), and the WT of IGF-1 sequences (IGF-1-WT) was amplified from the large white pigs by PCR. The process for constructing the vectors has been described in detail in our published protocols.[8] Finally, the expression vectors were successfully constructed and named pcDNA3.1-IGF-1-GCA, pcDNA3.1-IGF-1-GCG, pcDNA3.1-IGF-1-GCC, pcDNA3.1-IGF-1-GCT, pcDNA3.1-IGF-1-INT, and pcDNA3.1-IGF-1-WT, respectively (Figure 6A).

Microinjection of mice zygotes and embryo transplantation

Sexually mature female ICR mice (approximately 6 weeks old) underwent superovulation by intraperitoneal (i.p.) injection with 5 IU pregnant mare serum (PMS), 48 h after the injection. An i.p. injection of human chorionic gonadotropin (hCG) at a dose of 10 IU was given. Female mice were sacrificed the next day, and zygotes were collected. Transferred to the fresh M2 medium (Sigma) for washing 3−4 times, the oviducts were then collected and cultured in fresh M16 medium (Sigma) drops covered with paraffin oil for microinjection. A mixture of ABEmax mRNA (100 ng/μL) and sgRNA (50 ng/μL) was co-injected into the cytoplasm of pronuclear-stage zygotes.[76] Injected zygotes were cultured in M16 medium for *in vitro* analysis, and part of the two-cell stage zygotes was selected and transferred into the oviducts of pseudopregnant ICR female mice for offspring generation.

Genomic DNA extraction, PCR amplification, and genotyping

Injected embryos were collected at the blastocyst stage. Genomic DNA was extracted with an embryo lysis buffer (1% NP-40) at 56°C for 60 min and 95°C for 10 min in a Bio-Rad PCR amplifier and then subjected to Sanger sequencing. Genomic DNA from toes of newborn mice was extracted by TIANamp Genomic DNA Kit (TIANGEN) according to the manufacturer's protocols for PCR genotyping and sequencing. The isolated DNA was PCR amplified with 2× *Taq* Plus. All primers used for detection are listed in Table 10. Targeted sites were amplified from genomic DNA using Phusion polymerase (Thermo Fisher Scientific). The paired-end sequencing of PCR amplicons was performed by Sangon Biotech (Shanghai), using an Illumina MiSeq.

Off-target assay

The top five POTs for each sgRNA were predicted to analyze site-specific edits according to online design websites (https://zlab.bio/guide-design-resources), Cas-OFFinder (http://www.rgenome.net/cas-offinder/),[77,78] and CHOPCHOP (http://chopchop.cbu.uib.no/).[79] The off-target efficiency of each off-target site was estimated by sequence-matching degree and base mismatch. Then the PCR products of the POTs were sequenced. All primers for off-target assays were listed in Table 11.

Measurement of body-weight and body-size traits

Body weights and the tail lengths were recorded with an electronic path and vernier caliper every 3 days. F2-generation WT and He/Ho mutate mice were sacrificed at 8 weeks old by CO_2. The samples of heart, liver, spleen, lung, and kidney were collected and weighed for the viscera index, which was calculated as follows: viscera index = viscera weight (g)/body weight (g) × 100.[80] Both male and female

Table 10. *IGF-1* gene identification primers used to identify genotypes

Primers	Sequence (5′→3′)	PCR product (bp)	Extension time (s)	Annealing temperature (°C)
Primer-*IGF-1*-F	AACACCAGCCCATTCTGATTTG TTCACAGGTTTAGTCCTGGCTTTC	450	30	59.5
Primer-*IGF-1*-R				

Table 11. Mus-*IGF-1*-sgRNA primer sequences of potential off-target sequences

Potential off-target sequence	No. of mismatches	Genomic location	PCR primers
Mus-IGF-1-sgRNA1 (5′→3′)			
CCTGCATTCTGAGGGCAGCTCAG	2	chr1:74905373	F: ATCTCCAACTTGCTTCCTATGT
			R: CCTTCTGACCACCTTTCCTATT
CAGAGGTGCCCTCCGCAGGCAGG	3	chr6:145415851	F: GTCGGCCTGCTCTCTTATTT
			R: GGTTTCAGATTGGGAACTCTCT
CTGAGGTGCCCACAGAAGGCAGG	3	chr13:93931703	F: CAAGCCCAACTGGGATGATG
			R: TGGACAGCACCAATAAACTCAAAG
GCAGTCGCAGGGCCCCTCAGTGG	3	chr5:33443028	F: GTCTGCGATGTAGCGGGTGTT
			R: CTGTGCTGCGGAGCAAAGG
CCACTGCTCTGCCCTCTGAATGC	4	chr5:74214056	F: GGTGAGATGGCTTAAGAGGTAAA
			R: GACCAGCCCTGAAATCATACA
Mus-IGF-1-sgRNA2 (5′→3′)			
GACAGCTCAGAAAGGCATTGAGG	3	chr16:20965699	F: GGTGAGATGGTTCAGTGGTTAG
			R: GATGCTGCTGGGAGATGTT
GGCACAGAAGACAGGCATTGTGG	3	chr19:11447586	F: GTGGAATGAAACCTTTGTTAATCGG
			R: GCACTCAAACCAAGGTCAAAAGTCTA
GGCAGCACAGCCAGGCATTGAGG	3	chr6:55288621	F: CGGTCAGTGGCAAGAAGTTA
			R: ACTCCCACTCCAGGGATATT
GGCATCACAGACAGGCAATGGGG	3	chr15:72618648	F: AGCATCCCTCATTCCTTTCC
			R: GGCTCTACCCTTTCACATCTC
GAGACCTCAGACAGGCACTGAGG	3	chr8:91731460	F: GACCTGTTTGATTCCGCAAAG
			R: CCTCTGGTGTGGAAAGAGATTAC

No., number.

mice were included in the phenotype characterization with at least 5 samples from each group.

X-ray detection, H&E, and Alcian blue–alizarin red skeletal staining

X-ray autoradiography images of interest of whole-body skeletons in different genotypes of mice at 8 weeks old were taken using the YEMA Radiography System with a digital camera attached (Varian, Palo Alto, CA, USA) on X-ray film (ROTANODE; Toshiba, Tokyo, Japan). The images were taken at 125 kV with 400 mA exposure.[81]

The femur specimens from 8-week-old WT and mutant mice were harvested, skinned, and fixed in 4% paraformaldehyde for 2−4 days. After that, samples were dehydrated in ethanol solution, decalcified in 10% EDTA for 1 to 4 weeks, embedded in paraffin wax, and subsequently sectioned for slides.[82] The slides were stained with H&E and Alcian blue−alizarin red and then viewed under a Nikon TS100 inverted microscope.

Cell culture and cell transfection

PK-15 cells (porcine kidney cells) were cultured in DMEM (HyClone) supplemented with 5% fetal bovine serum (BI) and 1% penicillin-streptomycin (HyClone). Confluent PK-15 cells were transfected with 2.5 μg of pcDNA3.1-IGF-1-GCA, pcDNA3.1-IGF-1-GCG, pcDNA3.1-IGF-1-GCC, pcDNA3.1-IGF-1-GCT, pcDNA3.1-IGF-1-INT, and pcDNA3.1-IGF-1-WT, respectively, using Lipofectamine 2000 (Life Technologies, Invitrogen).

Quantitative real-time PCR and WB assay

Each total RNA was isolated from the liver tissues of five WT and He/Ho mutant mice at different growth and development stages (4 weeks, 6 weeks, and 8 weeks) and the pituitary tissues of 8-week-old mice. At the same time, protein solution was extracted by the liver tissue of 8-week-old mice. 36 h and 48 h after transfection, the PK-15 cells were lysed, and the total RNA and protein were extracted to detect the expression of FLAG tag. For the following stability test, PK-15 cells at 0, 1, 2, 4, and 6 h after transfection 24 h were collected and isolated total RNA and protein, respectively.

Total mRNA was reverse transcribed using the PrimeScript RT Reagent Kit with Genomic DNA Eraser (Takara). The amount and purity of the samples was determined with the Nanodrop ND-2000 spectrophotometer (Thermo Scientific). qRT-PCR reactions were performed using FastStart Universal SYBR Green Master (Rhone) on an ABI PRISM 7900HT thermocycler (Applied Biosystems, USA). Each sample was performed in triplicate, and data represented

Table 12. Primer information of qRT-PCR for IGF-1 and E peptides

Primers	Sequence (5′→3′)	PCR product (bp)	Extension time (s)	Annealing temperature (°C)
IGF-1-Ea-F	CCTCAGACAGGCATTGTGGAT	190	25	58.5
IGF-1-Ea-R	TTCTCCTTTGCAGCTTCGTTT			
IGF-1-Ec-F	GTGGACCGAGGGGCTTTTAC	215	25	58.5
IGF-1-Ec-R	ATGTACTTCCTTCTGAGTCTTGG			
IGF-1-F	TGGTGGATGCTCTTCAGTTCGT	179	25	58.5
IGF-1-R	TGCTTTTGTAGGCTTCAGTGGG			
GAPDH-F	TGGCAAAGTGGAGATTGTTGCC	156	25	58.5
GAPDH-R	AAGATGGTGATGGGCTTCCCG			
GH-F	TGCTGCTCATCCAGTCATGG	80	20	58.5
GH-R	CGAGGTGCCGAACATCAGG			
IGFBP3-F	TCTAAGCGGGAGACAGAATACG	91	20	58.5
IGFBP3-R	CTCTGGGACTCAGCACATTGA			

an average of at least three measurements. Ct values of the target genes were normalized with mean mRNA expression by the housekeeping gene. Data analysis was performed by the formula: $2^{-\Delta Ct}$ ($\Delta Ct = Ct_{IGF\text{-}1} - Ct_{\beta\text{-actin/GAPDH}}$). Primers used for qRT-PCR were listed in Table 12.

50 μg protein of each animal or 30 μg cell samples were loaded in a 6% agarose gel and separated on 15% SDS-PAGE gels and transferred to methanol-activated polyvinylidene difluoride membranes. The membranes were blocked for 1.5 h at room temperature in blocking buffer (5% non-fat dry milk dissolved in TBST buffer). Thereafter, the membranes were incubated with a rabbit polyclonal antibody raised against IGF-1 (1:2,000, ab9572; Abcam, USA) for tissue protein or a rabbit monoclonal antibody raised against FLAG (1:1,000, #14793; Cell Signaling Technology [CST], USA) for cell lysate overnight at 4°C, followed by incubation with the horseradish peroxidase-labeled secondary antibodies (1:5,000, anti-rabbit immunoglobulin G (IgG); Bioworld, USA) for 2 h at room temperature. Finally, the enhanced chemiluminescence plus WB detection system (Beyotime, China) was used for protein detection. The corresponding membranes containing intracellular proteins were stripped and detected by β-actin antibody (1:2,000; Bioworld, USA) in order to assess the correct protein loading. All membranes were developed with the ChemiDoc Touch Imaging System (Bio-Rad). The quantifications of the protein were analyzed using GenoSens Gel Analysis and ImageJ software (Rawak Software, Germany).

ELISA

WT and He/Ho mutant mice were anesthetized, and blood was collected by mice tail tip every 2 weeks from 4 weeks old to 12 weeks old. Plasma was obtained from tail blood samples of male and female mice at 4, 6, 8, and 12 weeks. Blood samples were allowed to clot for 2 h at room temperature before centrifuging for 20 min at 2,000 × *g* and then removing serum and store samples at −80°C for further detection. They were extracted in a final dilution of 1:500, and serum IGF-1 level was measured by the Quantikine Mouse/Rat IGF-I/IGF-1 Immunoassay Kit (MG100; R&D Systems, Minneapolis, MN, USA). A standard curve was generated for each set of samples assayed. This kit detects total rodent IGF-1, and there is no crossreactivity or interference with IGF-2 or IGFBPs. Data were acquired in duplicate with a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm.

In vitro translation assay

To further examine the effect of synonymous codons on IGF-1 protein synthesis, the Retic Lysate IVT Kit (Thermo Scientific, USA) was used to evaluate the protein synthesis efficiency of different IGF-1 expression plasmids encoded by various synonymous codons. 7.5 μg RNA template was isolated from PK-15 cells and quantitated by the Nanodrop ND-2000 spectrophotometer (Thermo Scientific). Specific amounts of the indicated reagents (20× translation mix, unlabeled methionine, retic lysate, RNA template) were added in the order according to the instructions, and Retic Lysate IVT reactions were assembled. Each tube was mixed and centrifuged briefly to collect the reaction at the bottom and then incubated at 30°C for 60 min in a water bath. After that, the *in vitro* translation products were placed on ice to stop the reactions. Finally, translation products were analyzed by ELISA.

Stability assay

Confluent PK-15 cells were transfected with each IGF-1 expression plasmid with different synonymous codons in a single 10-cm dish. After 6 h, transfected cells were split in a 6-well plate in triplicate at around 50% confluence. 24 h after transfection, ActD (25 μg/mL) and actinomycin (CHX; 40 μg/mL) were added to the serum-free culture media to interfere with mRNA transcription and protein biosynthesis. Then the cells were harvested at 0, 1, 2, 4, and 6 h for total RNA and protein extraction. The total RNA was used to detect the mRNA stability, and the protein was prepared for the protein stability monitor, respectively.[8]

Cell proliferation assay

To detect whether IGF-1 with different synonymous codons affects its function, cell proliferation assays were performed using the Cell

Counting Kit-8 (CCK-8; Dojindo, Japan). IGF-1 expression vectors with different synonymous codons were transfected into PK-15 cells, respectively. 24 h later, they were inoculated in 96-well plates at an initial density of 5,000 cells/well. Then, 10 μL of the CCK-8 reagent was added at 0, 24, 48, and 72 h after cell attachment and incubated at 37°C for 50 min. Then, the absorbance at a wavelength of 450 nm was detected using a microplate reader (Eon; BioTek, USA).

Bioinformatics and statistical analysis

Based on the gene database from NCBI (https://www.ncbi.nlm.nih.gov/snp) and the genome database from Ensembl (http://grch37.ensembl.org/index.html), the homology of the *IGF-1* coding sequence region among various species was detected from the published DNA sequences. gws and genome-wide association study (GWAS) database were used for online alignment and then to investigate the codon preference discipline at *IGF-1* c.258bp in diverse mammals with different body-size types. Furthermore, considering that the synonymous mutation encodes Ala, the synonymous codon usage frequency of Ala was analyzed according to the codon usage database (http://www.kazusa.or.jp/codon). To detect the potential mRNA secondary structure, we predicted the mRNA folding form and the minimum free energy by using the Mfold web server (http://www.unafold.org/). Primers in this study were designed using primer Premier Software version 5.0 (Premier Biosoft International) and synthesized by Jilin Comate Bioscience.

All of the replicate experiments were repeated for at least three individual determinations. Data obtained are represented as mean ± SEM, and the comparison was analyzed with Student's unpaired t test or one-way ANOVA as appropriate. All statistical tests were performed using GraphPad Prism 8.0.2 (GraphPad Software, La Jolla, CA, USA). All statistical tests were two-tailed, and p values <0.05 were considered as statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.08.007.

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AUTHOR CONTRIBUTIONS

All authors certify that they have participated in the work. S.W., Y.C., and L.H. participated in the study conception and design. S.W., Y.X., Y.G., Z.W., P.X., and J.S. performed experiments. Moreover, S.W., Y.X., C.W., T.F., and G.L. analyzed the data. S.W. and S.L. interpreted the data and then wrote the manuscript. Finally, L.H. and S.L. revised the manuscript.

DECLARATION OF INTERESTS

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work. There is no professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the position presented in the manuscript.

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