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ORIGINAL ARTICLE

# Expression mechanisms of mir-486-5p and its host gene sANK1 in porcine muscle

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## Abstract

**Background** *MiR-486-5p* has been identified as a crucial regulator of the PI3K/AKT signalling pathway, which plays a significant role in skeletal muscle development. Its host gene, *sANK1*, is also essential for skeletal muscle development. However, the understanding of porcine *miR-486-5p* and *sANK1* has been limited.

**Methods and results** In this study, PCR analyses revealed a positive correlation between the expression of *miR-486-5p* and *sANK1* in the longissimus dorsi muscle of the Bama mini-pig and Landrace-pig, as well as during myoblast differentiation. Furthermore, the expression of *miR-486-5p/sANK1* was higher in the Bama mini-pig compared to the Landrace-pig. There was a total of 18 single nucleotide polymorphisms (SNP) present in the *sANK1* promoter region. Among these SNPs, 14 of them resulted in alterations in transcription factor binding sites (TFBs). Additionally, the promoter fluorescence assay demonstrated that the activity of the *sANK1* promoter derived from the Bama mini-pig was significantly higher compared to Landrace-pig. It is worth noting that ten regulatory SNPs have the potential to influence the activity of the *sANK1* promoter. A nuclear mutation A-G located at position − 401 (relative to the transcription start site) in the Bama mini-pig was identified, which creates a putative TFB motif for *MyoD*.

**Conclusions** The findings presented in this study offer fundamental molecular knowledge and expression patterns of *miR-486-5p/sANK1*, which can be valuable for gaining a deeper understanding of the gene's involvement in porcine skeletal muscle development, and meat quality.

**Keywords** *miR-486-5p* · *sANK1* · Skeletal muscle · Pig

## Introduction

Pig (*Sus scrofa*) is an important animal for meat production as well as model organisms for biomedical research [1]. The Bama mini-pig, a breed of domestic dwarfism originating from Guangxi province in China, exhibits early maturation and possesses a smaller body size and higher fat content, but a smaller muscle mass in the carcass when compared to Landrace pigs. The observed variations in carcass phenotype between these two breeds can be attributed not only to differences in growing environment and physiological metabolism, but also to genetic disparities that significantly influence skeletal muscle development and the process of fat deposition.

*MiR-486-5p* plays a crucial role as a modulator of the PI3K/AKT signaling pathway, which is intricately involved in the process of skeletal muscle development. Additionally, the host gene *sANK1* is deemed indispensable for the proper development of skeletal muscle [2]. The bioinformatics analysis revealed the involvement of miR-486-5p in the regulation of fat deposition and lipid differentiation [3]. The Porcine ankyrin 1 gene (*ANK1*) is a gene of significant potential and functionality, situated on chromosome 17 (*SSC17*) in close proximity to multiple quantitative trait loci (QTL) associated with meat quality traits [4–6]. The presented evidence supports the notion that *miR-486* and

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*sANK1* exhibit potential and functionality in the context of skeletal muscle development and meat quality traits. Our preliminary investigation further substantiates the existence of a noteworthy disparity in *miR-486-5p* expression within the skeletal muscle of Bama pigs and Landrace-pigs [7]. However, the underlying factors contributing to the observed variations in *miR-486-5p* expression between Bama pigs and Landrace remain unidentified.

Approximately 37% of the identified mammalian miRNAs are situated within the introns of protein coding genes, commonly referred to as host genes, thereby establishing a connection between their expression and the promoter-driven regulation of the host gene [8]. The transcription of *MiR-486-5p* occurs from an intron within the Ankyrin-1 gene, which is transcribed into two isoforms: a longer isoform enriched in erythroid cells, and a shorter isoform enriched in heart and skeletal muscle cells [2]. Intronic microRNAs typically exhibit coordinated expression with their respective host gene messenger RNA, suggesting that they are commonly derived from a shared transcript [9]. The expression levels of *MiR-486-5p* and its host gene *sANK1* were found to be abundant in mouse muscles, exhibiting a gradual up-regulation throughout the process of myoblast differentiation [10]. The transcription of *MiR-486-5p* was regulated by an alternative promoter located within intron 40 of the *Ankyrin-1* gene. This regulation was mediated by *SRF*, *MRTF-A*, and *MyoD* [2]. In our prior investigation, we identified three transcripts of *sANK1* genes in Bama mini-pig, specifically encoded by exon 39a and the final four exons (40–43) of ankyrin-1 [11]. However, it is currently unknown whether any studies have been conducted on the expression patterns and regulation mechanism of *miR-486/sANK1* in the development of porcine skeletal muscle.

In this study, we conducted an investigation into the expression levels of *miR-486* and *sANK1* during the development of porcine skeletal muscle and myoblast differentiation. We also examined the SNP sites and of the *sANK1* promoter, and found evidence suggesting that ten regulatory SNPs may impact the activity of the *sANK1* promoter. Our findings indicate that a nuclear mutation A-G at position −401 (relative to the transcription start site) in the Bama mini-pig creates a potential transcription factor binding motif for *MyoD*, which may contribute to the increased expression of *miR-486-5p/sANK*.

## Materials and methods

### Animal and sample collection

The procedures for all animal studies were conducted in accordance with the guidelines set forth by national and local animal welfare organizations. The animals used in this study were sourced from the Bama mini-pig breeding center at Guangxi University. Specifically, the longissimus dorsi muscles were isolated from two male and two female pigs at each time point, spanning five postnatal stages of both Bama mini-pig and Landrace breeds, namely 1, 30, 60, 90, and 180 days old (referred to as 1D, 30D, 60D, 90D, and 180D, respectively). Furthermore, a total of fourteen tissues, encompassing the heart, liver, spleen, lungs, kidney, stomach, intestine, aorta, brain, biceps femoris, longissimus dorsi muscle, pancreas, ovary, and bladder, were procured from adult sows. To guarantee the extraction of RNA of utmost quality, all harvested tissues were promptly snap-frozen in liquid nitrogen within a thirty-minute timeframe.

### Total DNA and RNA isolation and cDNA synthesis

Porcine tissue DNA ($n = 4$ pig/group) was isolated using the Blood and Tissue DNA Extraction Kit (TIANGEN, China). For RNA extraction, porcine tissue was digested in RNAiso reagent following the manufacturer's instructions (Takara, Japan). The RNA concentrations were determined using a UV spectrophotometer (Thermo, NanoDrop 1000), and the OD260/OD280 ratio was confirmed to be between 1.8 and 2.0. To assess the integrity of the RNA, a 1% agarose gel stained with ethidium bromide was utilized. The RNA was then aliquoted and stored at − 80 ℃ until further use.

In the process of miRNA cDNA synthesis, a total of 2 µg of treated total RNA from each independent sample was subjected to polyadenylation with ATP by poly(A) polymerase (PAP). Subsequently, reverse transcription was performed using PrimeScript RTase at 37 ℃ for 60 min, followed by enzyme inactivation at 85 ℃ for 5 s in a final volume of 20µL reaction mixture, adhering to the guidelines provided in the instruction manual of the SYBR®PrimeScript™ miRNA RT-PCR Kit (Takara, Japan). For the synthesis of mRNA cDNA, a total of 2 ug of RNA from each independent sample was subjected to DNA removal and reverse transcription using Prime Script RT Enzyme Mix1. This process involved incubation at 42 ℃ for 15 min followed by enzyme inactivation at 85 ℃ for 5 s, all within a final volume of 20 µl reaction mixture. The procedure followed was that of the PrimeScriptTMRT reagent Kit with gDNA Eraser (TaKaRa, Japan).

### Semi-quantitative RT-qPCR

The RT-qPCR amplification process was conducted under the specified cycling conditions, which included an initial denaturation step at 94 ℃ for 30 s, followed by 35 cycles of denaturation at 94 ℃ for 30 s, annealing at 60 ℃ for 30 s, and extension at 72 ℃ for 30 s. The resulting PCR products

were observed and analyzed on 1.2% agarose gels. Expression levels were determined by comparing them to the reference gene β-actin.

## Real-time PCR

Real-time PCR was conducted using SYBR Premix Ex Taq II (Takara, Japan) and a LightCycle480 real-time PCR system (Roche Diagnostics, Germany) in accordance with the manufacturer's instructions. Each assay consisted of a 20μL final volume, comprising 10μL of SYBR Premix Ex Taq II, 200 nM of each primer, and 5μL of a 1:100 dilution of the cDNA template. The reactions were carried out in a 96-well optical plate, with an initial incubation at 95 ℃ for 5 min, followed by 40 cycles of 95 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 30 s. A final cycle of 95 ℃, 65 ℃, and 95 ℃ was performed for melting curve analysis. The melting curve analysis was conducted in every assay to identify any instances of non-specific amplifications. Each reaction was replicated three times, and each run encompassed reactions with negative controls (no template) and positive controls for each gene.

The RT-qPCR primers utilized in this study can be found in Supplemental Table 1. Porcine U6 small nuclear RNA was employed as a normalization factor for *miR-486*, while β-actin served as an internal control for *sANK1* and *GFP*. The gene relative expression levels were determined using the $2^{-\triangle\triangle Ct}$ method, and the resulting data were subjected to statistical analysis using SPSS 17.0. The mean values ± standard error ($n = 4$) was reported to express the experimental results. The statistical significance of differences between groups was assessed using a T-test, with $P$-values < 0.05 indicating significance and $P$-values < 0.01 indicating extreme significance. To measure the expression correlation between *miR-486* and its host gene *sANK1*, Pearson correlation analysis was employed.

## Promoter SNP discovery and transcriptional effects of regulatory SNP

The transcription start site (TSS) of the porcine *sANK1* gene was determined using a computational prediction approach available at http://www.fruitfly.org/seq_tools/promoter.html. To gain insight into the SNP of the porcine *sANK1* promoter, a 933 bp segment of the promoter region (-729 ~ + 202 bp relative to the TSS) of the *sANK1* gene was amplified and sequenced from genomic DNA pools of Bama-mini pig, Landrace, and F1 crossbred populations, each consisting of 20 individuals. Gene-specific primers (Supplemental Table 1) were designed according to the upstream region of the predicted TSS for *Sus scrofa* Ankyrin-1-like transcript variant. The sequence was aligned and analyzed using DNA star software. In order to identify SNPs that may impact the promoter elements, including potential binding sites for muscle-specific transcription factors, the promoter region of *sANK1* was screened for the presence of putative selective using four in silico prediction tools (TESS: http://www.cbil.upenn.edu/tess, AliBaba2: http://www.gene-regulation.com/pub/programs/alibaba2/index.html, MATCHTM: http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi and MatInspector: http://www.gsf.de/biodv/matinspector.html).

## Plasmid construct

To investigate the activity of the *sANK1* promoter, a reporter vector containing the sANK promoter was generated. This was achieved by amplifying two distinct lengths of the promoter DNA fragment (-1792 ~ + 381 bp, -945 ~ + 220 bp to TSS) from Bama-mini pig and Landrace genomic DNA, using gene-specific primers (Supplemental Table 1). Subsequently, the amplified products were inserted upstream of the EGFP ORF in the pEGFP-1 vector, utilizing the *BamH* / and *Hind* /// restriction sites. The computational prediction was utilized to determine the anticipated TSS and the corresponding accession is situated on chromosome 17 according to the pig Aug.2011 (SGSC Sscrofa10.2/susScr3) assembly in the UCSC browser. The verification of all sequences was conducted through DNA sequencing.

## Cell culture and transfection

C2C12 (mouse myoblast), PK15 (porcine Kidney-15), and PEF(porcine ear fibroblasts) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at a temperature of 37 ℃ and a $CO_2$ concentration of 5%. The induction of myogenic differentiation in C2C12 myoblasts to myotubes was achieved by substituting the medium with DMEM supplemented with 2% horse serum for the duration specified in the figure legends. For the cell transfection experiments, C2C12, PK15, and PEF cells were cultured in growth medium until they reached approximately 70–80% confluency. These cells were then transfected with plasmids, namely pEGFP-BP1, pEGFP-BP2, pEGFP-LP1, and pEGFP-LP2, using the LTX transfection reagent (Invitrogen) as per the manufacturer's instructions. In addition, a negative control (the promoter-less plasmid pEGFP-1) and two positive controls (containing pEGFP-C1 and pEGFP-N1) were included in the experiment. Each transfection was performed in triplicate, and after 48 h, RNA was extracted for subsequent experiments.

## Result

### The expression patterns of mir-486-5p and host sANK1 gene in porcine muscle

In order to investigate the co-expression relationship between *miR-486* and the host ANK1 gene, the *miR-486-5p* precursor and its host *sANK1* gene were amplified using PCR with genomic DNA and cDNA from muscle tissue, respectively. The obtained results demonstrated that the miR-486-5p precursor could be amplified from both the cDNA and DNA templates, while Ex42 ~ Ex43 of *sANK1* could be amplified solely from the cDNA template (Fig. 1a). To gain insight into the expression patterns of *miR-486-5p* and *sANK1* in porcine tissues, RT-qPCR was employed to amplify *sANK1* and *miR-486-5p* from 14 different tissues of Bama mini-pig. The findings of this study indicate that *miR-486-5p* exhibited expression in various organs and tissues, including the heart, aorta, lung, longissimus dorsi muscle, spleen, stomach, intestine, pancreas, biceps femoris, and bladder. Conversely, the *sANK1* gene demonstrated expression solely in the heart, longissimus dorsi muscle, and biceps femoris, as depicted in Fig. 1b.

### There exists a correlation between the expression levels of *miR-486-5p* and *sANK1*

The expression patterns of *miR-486-5p* and *sANK1* were analysed in porcine skeletal muscle during postnatal development (Days 1, 30, 60, 90, and 180) in Bama mini-pig and Landrace-pig using RT-qPCR. It was observed that the expression patterns of *miR-486* and *sANK1* genes exhibited a notable increase in accordance with the progression of daily ages. In the Bama mini-pig, the correlation coefficient was determined to be $R^2 = 0.52$ ($P = 0.168$), while in the Landrace-pig, the correlation coefficient was $R^2 = 0.49$ ($P = 0.190$) (Fig. 2a). Additionally, the expression patterns of *miR-486-5p* and *sANK1* were assessed in C2C12 cells during myoblast differentiation using RT-qPCR. The findings revealed a significant increase in the expression levels of *miR-486-5p* and *sANK1* as the differentiation process progressed in C2C12 cells, with a strong positive correlation coefficient of $R^2 = 1.00$ ($P < 0.05$). (Figure 2b and c). Furthermore, the levels of *miR-486-5p* and *sANK1* expression in Bama mini-pig were found to be significantly elevated compared to Landrace pigs on day 60, day 90, and day 180 (Fig. 2d and e).

### The promoter of the porcine miR-486/sANK1 gene was cloned and subjected to analysis

To comprehend the configuration of porcine *miR-486/sANK1* promoters, a 4245 bp segment from intron 39 of the porcine *sANK1* gene was obtained from the Ensembl database. The A base of the translation initiation codon ATG within the 39a exon was assigned the position of "+1". The encompassing sequence spanned 3445 bp upstream and 800 bp downstream, resulting in a total length of 4245 bp. Initially, the selected fragments underwent CpG island analysis. The analysis conducted using the Methprimer online program revealed the presence of a single CpG island within the − 869 to -768 range, satisfying the criteria of having a GC content exceeding 50% and an Obs/Exp ratio surpassing 0.6. The analysis of the SMS CpG program revealed the presence of a single CpG island within the − 1008 to -590 interval. Similarly, the CpGProD program analysis identified one CpG island within the − 239 to + 282 interval. Additionally, the First EF website predicted the presence of one CpG island within the + 70 to + 272 interval. Furthermore, the CpG plot prediction results indicated the existence of two CpG islands within the − 985 to -677 interval and the − 60 to + 61 interval. Subsequently, promoter prediction analysis was conducted on the selected fragments. The NNPP website (http://www.fruitfly.org/seq_tools/promoter.html) displayed the promoter region spanning from − 237 to -186, with the TSS situated at -197. The initial EF online site prediction yielded the most likely promoter region to be -417 to + 153 bp. Promoter Scan identified three promoter core regions, although their scores ranged from 55 to 69.5 points, indicating relatively low confidence (Fig. 3a). Based

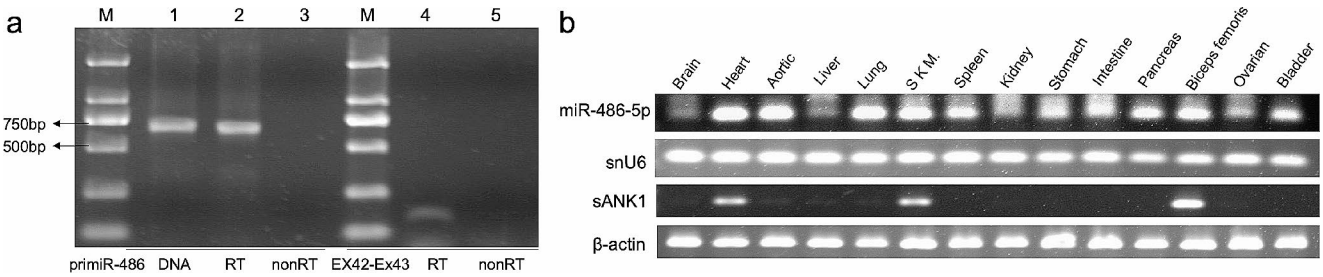



**Fig. 1** The expression patterns of *miR-486-5p* and the host *sANK1* gene in porcine muscle were examined. (a) The initial transcription of porcine *miR-486-5p* and the transcription of its host gene *sANK1* were identified to be co-expressed. (b) The expression patterns of *miR-486-5p* and the host *sANK1* gene were analysed in 14 different tissues of Bama mini-pig. *snU6* and *β-actin* were used as controls for RT-qPCR.

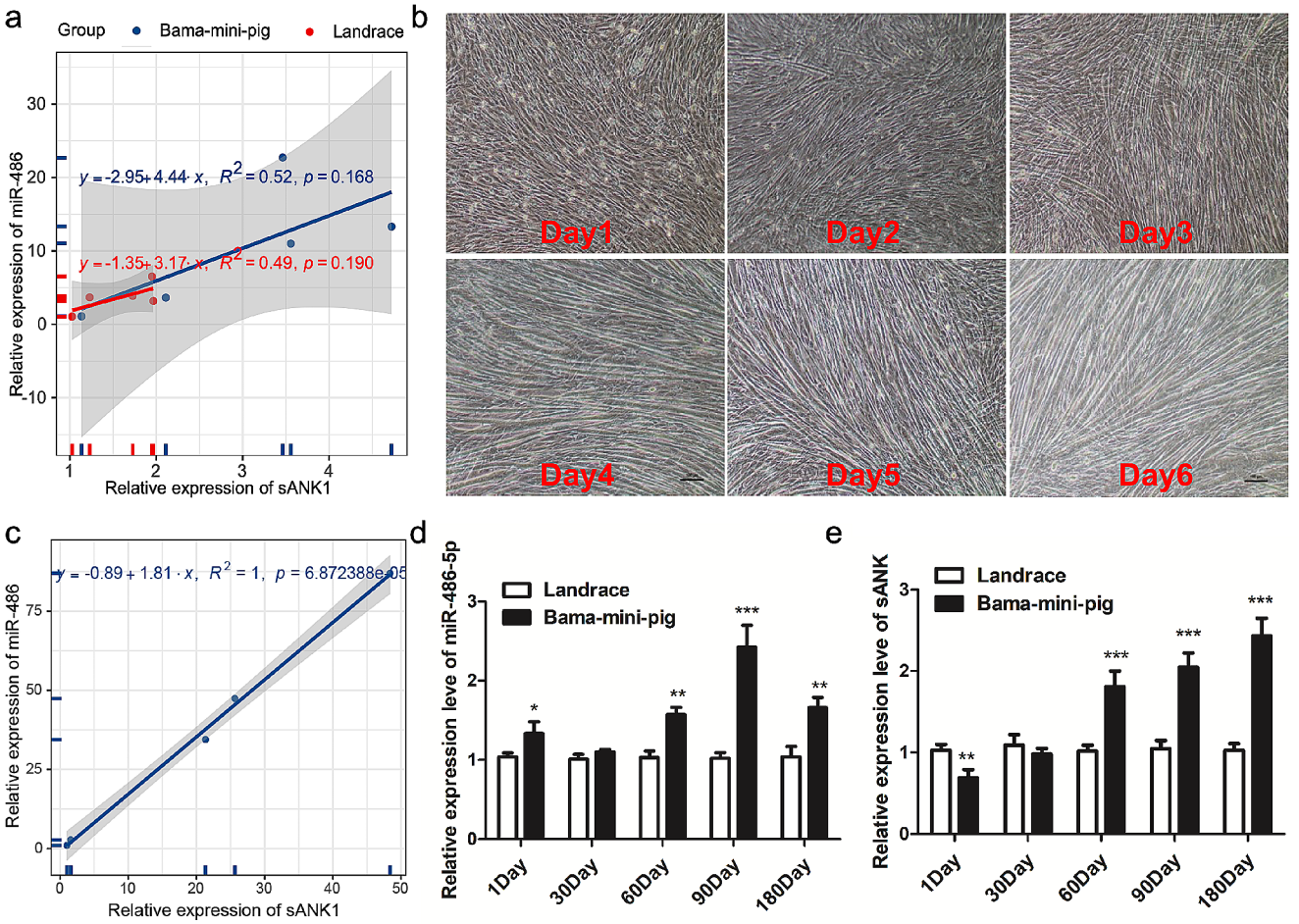



**Fig. 2** There exists a positive correlation between the expression levels of *miR-486-5p* and *sANK1*. (**a**) The Pearson correlation coefficient was employed to calculate the associations between *miR-486* and *sANK1* expression levels during postnatal development stages in skeletal muscle of Bama mini-pig and Landrace pigs. (**b**) The cellular morphology of C2C12 cells at various stages of differentiation. (**c**) The Pearson correlation coefficient was employed to calculate the correlations between *miR-486* and *sANK1* expression during the differentiation process of C2C12 myoblasts. (**d**) The expression of *miR-486-5p* was observed in postnatal porcine skeletal muscle of Bama mini-pig and Landrace using RT-qPCR. (**e**) The expression of sANK1 was assessed using RT-qPCR in postnatal porcine skeletal muscle of Bama mini-pig and Landrac

on the typical positioning of the mammalian core promoter, which is commonly situated approximately 100–200 bp upstream of the transcription start site, it is postulated that the −417 to +153 bp region potentially serves as the core promoter for the porcine *sANK1* gene.

To analyse the transcription factor binding site of porcine *miR-486/sANK1* promoters, a multiple alignment was conducted using ClustalX. This alignment involved a 1000 bp sequence located upstream of the translation initiation codon ATG of the porcine *sANK1* promoter gene, along with other available ANK1 homologues on NCBI. Subsequently, transcription factor binding site analysis was performed using various tools such as MATCH, AliBaba2, Patch, MatInspector, TESS, TFSEARCH, and other websites or software. The obtained results predicted the presence of *MyoD*, *TEF*, *myogenin*, and *Myf-3* transcription factors. Several transcription factors, including *Pax-2*, *Pax-5*, *Pax-8*, *YY1*, *Sp1*, *Sox2*, *AP2*, *C/EBPalp*, *PPAR-α*, *GR*, *NF-1*, *USF*, *NF-kappa*, *CREB*, and *REB1*, are abundantly present in muscle tissue. Notably, *MyoD*, *myogenin*, *myf-3*, *Pax-2*, *Pax-5*, *Pax-8*, *YY1*, and other factors fall within this category of muscle-enriched transcription factors (Fig. 3b).

To gain a deeper understanding of the differentiation between *miR-486/sANK1* promoters in Bama-mini pigs and Landrace species, specific primers were developed to amplify a sequence ranging from −729 to +202 bp. The findings revealed the presence of a single TSS located at -197 site (indicated by yellow markers) upstream of the new exon 1. Additionally, four potential TATA boxes were identified at positions 30 bp, 233 bp, 532 bp, and 600 bp upstream of the TSS: TAAAATTA, TTATTTT, TTAAA TA, and TTTAA. The promoter region exhibited a high G+C content of 62.17% and was notably enriched in guanine clusters. Furthermore, a multitude of TFBSs were

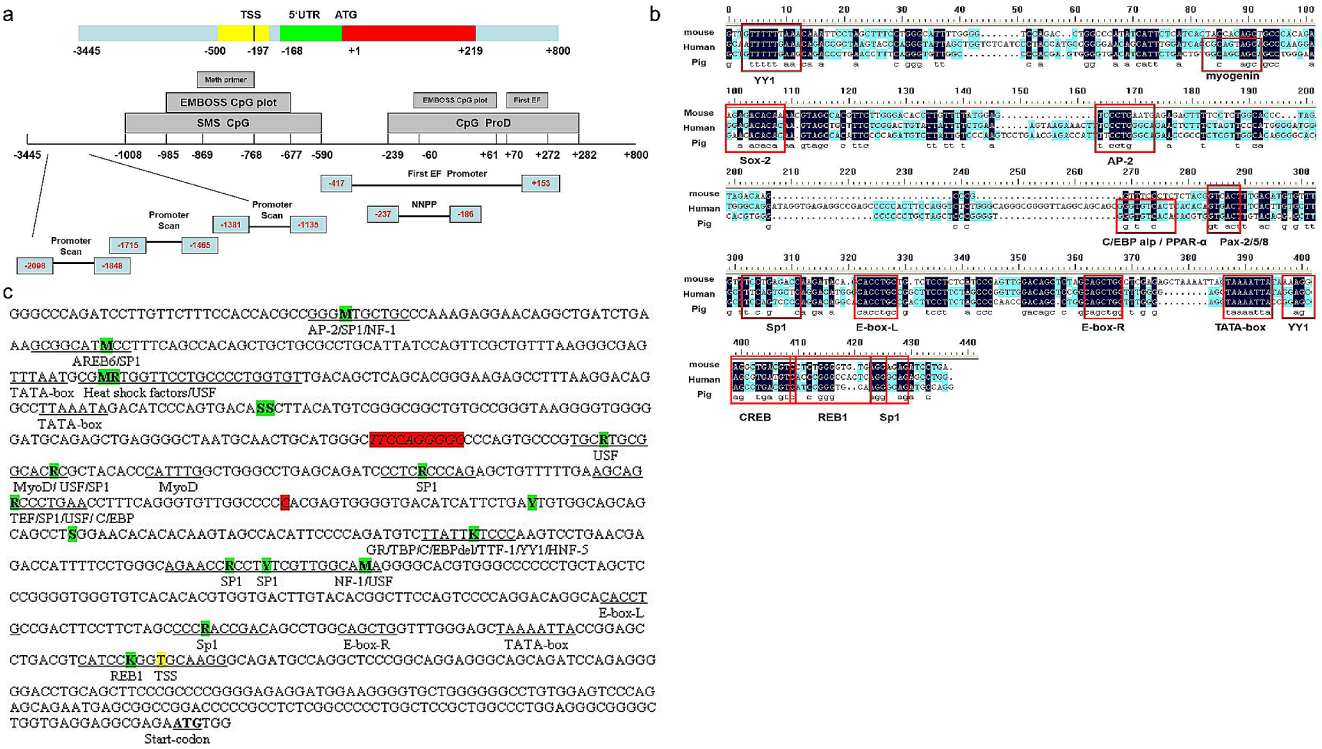


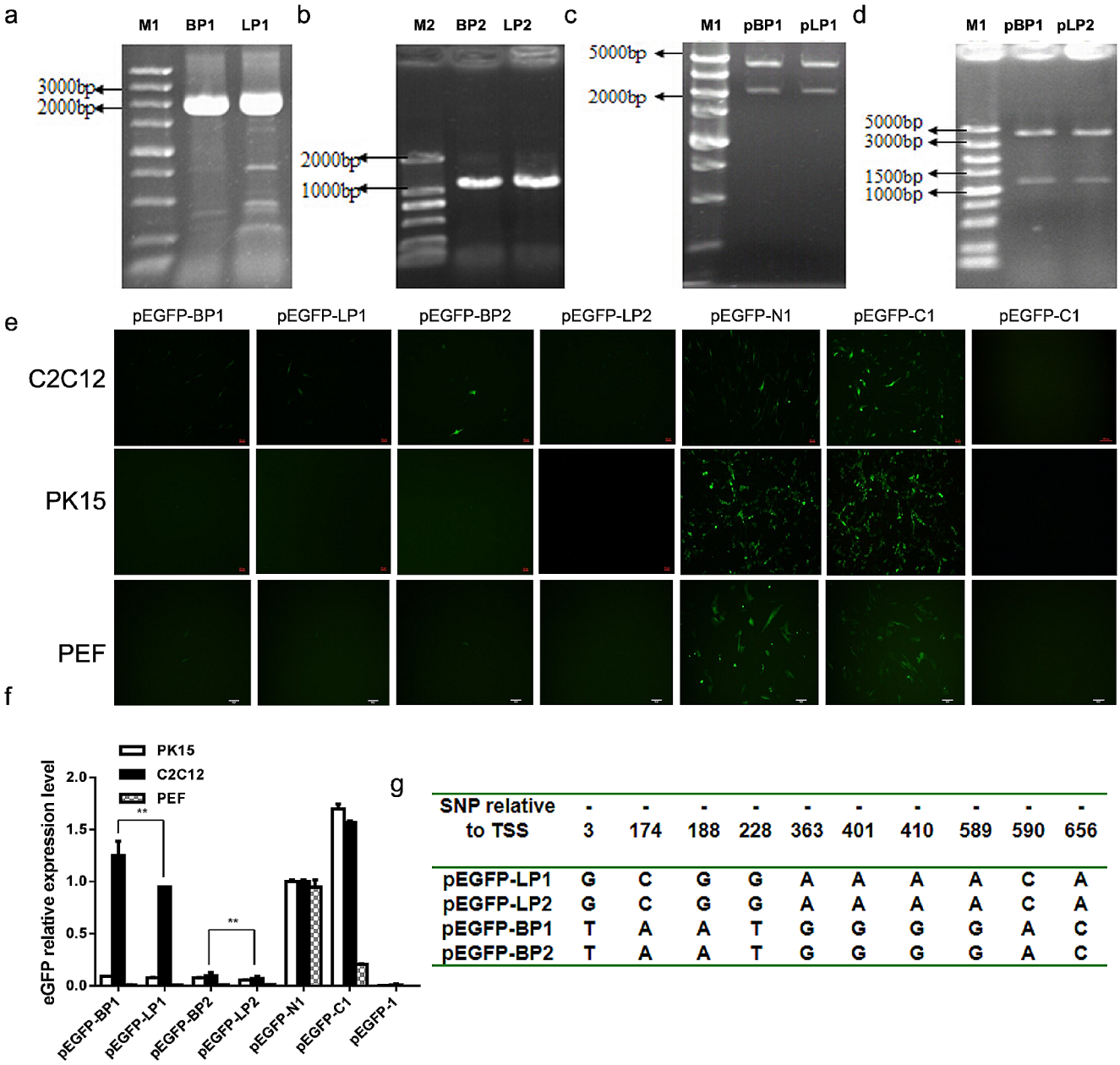

**Fig. 3** The promoter region of the porcine *miR-486/sANK1* gene was cloned and subjected to analysis. (**a**) The integrated graphics depict the prediction of the *sANK1* gene promoter and its associated CpG island. (**b**) The present study involves the examination of homology and transcription factor binding site analysis of the *sANK1* promoter. (**c**) The nucleotide sequence of the regulatory region preceding the translation initiation site of porcine *sANK1* is presented. The presence of two TSS, the start codon (ATG), and SNPs within the TFBs are indicated. The IUPAC-IUB symbols for nucleotide nomenclature, including M: (A/C), R:(A/G), S:(C/G), Y:(C/T), and K: (G/T), are highlighted in bold

identified within the promoter region, including two conserved E-boxes that impart responsiveness to *MyoD*, situated at positions − 46 bp and − 87 bp, respectively (Fig. 3c). The statistical analysis unveiled a total of 18 SNP sites, represented by green markers, within the specified region of the *sANK1* gene promoter. Additionally, two deletions, indicated by red markers, were observed in this same region. Furthermore, several transcription factors binding sites were identified, including two E-boxes located at positions − 46 bp and − 87 bp (Fig. 3c).

To ascertain whether SNPs had an impact on the creation or elimination of hypothetical binding motifs for transcription factors, thereby qualifying as regulatory SNPs. We conducted an analysis of the SNPs present within the TFBs of *sANK1* regulatory regions. Through sequencing a region of 933 bp surrounding the TFBs in Bama-mini pig, Landrace, and the F1 crossbred population (Landrace♂×Bama-mini pig♀). We identified a total of eighteen SNPs, consisting of eleven newly discovered SNPs and seven previously documented SNPs. Transcription factor binding motifs that corresponded with SNPs were predicted using TESS, AliBaba2, MATCH, and MatInspector tools. As indicated in Supplemental Table 2, a total of 14 SNPs were found to result in alterations in TFBs. These putative regulatory SNPs may potentially impact the binding of muscle-specific transcription factors.

## The activity of the porcine sANK1 promoter is regulated by a regulatory SNP

In order to confirm the regulatory SNPs that impact the activity of the sANK1 promoter in Bama mini-pig and Landrace, two distinct fragments (-1792 ~ + 381 bp, -945 ~ + 220 bp relative to the TSS) of the promoters from both breeds were cloned and inserted into the pEGFP-N1 vector to generate the corresponding expression vectors, namely pEGFP-BP1, pEGFP-BP2, pEGFP-LP1, and pEGFP-LP2 (Fig. 4a-d). The subsequent measurement of promoter activity was conducted using fluorescence intensity and GFP expression. The findings indicated that pEGFP-BP1, pEGFP-LP1, pEGFP-BP2, and pEGFP-LP2 were able to drive GFP expression in C2C12 cells, but not in PK15 and PEF cells. Notably, pEGFP-BP1 and pEGFP-LP1 demonstrated significantly higher levels of GFP expression compared to pEGFP-BP2 and pEGFP-LP2 in C2C12 cells. Furthermore, pEGFP-BP1 exhibited significantly higher GFP expression




| SNP relative to TSS | - 3 | - 174 | - 188 | - 228 | - 363 | - 401 | - 410 | - 589 | - 590 | - 656 |
|---|---|---|---|---|---|---|---|---|---|---|
| pEGFP-LP1 | G | C | G | G | A | A | A | A | C | A |
| pEGFP-LP2 | G | C | G | G | A | A | A | A | C | A |
| pEGFP-BP1 | T | A | A | T | G | G | G | G | A | C |
| pEGFP-BP2 | T | A | A | T | G | G | G | G | A | C |

**Fig. 4** The activity of the porcine *sANK1* promoter is regulated by a regulatory SNP. (a-d) Construction of promoter vector. Agarose gel electrophoresis of the *sANK1* promoter PCR products, BP1, LP1 (a) and BP2, LP2 (b) represent *sANK1* promoter from Bama mini-pig and Landrace-pig, respectively. Identification of vector by double enzyme digestion (c and d). (e) The fluorescence intensity exhibited by cells that have undergone transfection with a promoter. (f) The expression of green fluorescent protein (GFP) was observed in various cell types that were transfected with distinct promoter sequences. (g) Various attributes of the promoter

than pEGFP-LP1, while pEGFP-BP2 exhibited significantly higher GFP expression than pEGFP-LP2 in C2C12 cells (Fig. 4e and f). Collectively, these findings suggest that the *sANK1* promoter exhibits muscle-specificity, and the −1792~+381 bp fragment, which contains crucial elements, is sufficient for its expression in muscle tissues. Additionally, the *miR-486-5p/sANK1* promoter activity in Bama mini-pig surpasses that of Landrace-pig. In order to comprehend the underlying reasons for the elevated *sANK1* promoter activity in Bama mini-pig compared to Landrace-pig, we conducted a comprehensive analysis of the promoter's characteristics across different breeds. The findings indicate that ten regulatory SNPs may have an impact on the activity of the *sANK1* promoter. Additionally, a nuclear mutation A-G at position −401 (relative to the TSS) in Bama mini-pig is observed, which potentially generates binding motifs for the *MyoD* transcription factor. This mutation

may potentially contribute to the increased expression of *miR-486/sANK1*(Fig. 4g).

## Discussion

Future research directions may also be highlighted. The role of *miR-486-5p* and its host gene *sANK1* in porcine skeletal muscle development and meat traits is of significant importance. However, our current understanding of the expression mechanisms of *miR-486-5p* and *sANK1* in porcine skeletal muscle is limited. In this study, we aimed to investigate the expression pattern of *miR-486-5p* and *sANK1* and elucidate novel mechanisms of their expression in porcine muscle. Our findings revealed that the *miR-486-5p* precursor can be amplified from both cDNA and DNA templates, while Ex42∼Ex43 of *ANK1* can be amplified solely from the cDNA template. The data presented in this study indicate a co-expression of *miR-486-5p* initial transcription and host gene *sANK1* transcription. Additionally, *miR-486-5p* was found to be present in a majority of tissues, whereas *sANK1* was only detected in the heart, longissimus muscle, and biceps femoris. This finding suggests that *sANK1* plays a crucial role in muscle development. Furthermore, a significantly higher level of *miR-486* and a muscle-specific expression of the *sANK1* gene were observed in skeletal muscle and cardiac tissues compared to other tissues examined, indicating a dependence on muscle tissues for their expression. The distribution patterns of *miR-486* and *sANK1* in porcine tissues observed in our study align with the distribution patterns previously reported in mouse tissues [2]. Therefore, based on our research findings, it can be inferred that *miR-486* and its host gene *sANK1* exhibit co-expression in porcine muscle tissues.

The co-expression patterns of *miR-486* and *sANK1* were observed during the differentiation of C2C12 myoblasts, the expression patterns in porcine were not reported in previous studies. To enhance comprehension of the involvement of *miR-486/sANK1* and investigate the expression patterns of *miR-486* and *sANK1* during postnatal skeletal muscle development in pigs, a comparative analysis of *miR-486* and *sANK1* expression levels was conducted across five developmental stages in Bama mini-pig and Landrace pigs. The findings of our research demonstrate a noteworthy pattern wherein the expression levels of *miR-486* and *sANK1* exhibit a gradual increase throughout the process of C2C12 myoblast differentiation. Furthermore, this gradual up-regulation is observed during postnatal skeletal muscle development in both breeds, thereby affirming a positive association between the expression of *miR-486* and *sANK1*. Additionally, it is plausible to hypothesize that *miR-486* and *sANK1* may share a common transcript, regulated at the transcriptional level, within porcine skeletal muscle. This regulation may be facilitated by the promoter of porcine *sANK1*. In contrast, the accumulation rate of *sANK1* was considerably slower than that of *miR-486* throughout postnatal skeletal muscle development and myoblast differentiation. This suggests that despite sharing the same transcript, their expression stability is still subject to regulation by other factors, such as transcript factors.

At the same time points, various porcine breeds demonstrate distinct gene expression phenotypes [12]. The findings of our study indicate a statistically significant elevation in the expression level of *miR-486-5p* in the skeletal muscle of Bama mini-pig compared to Landrace pigs at day 60, day 90, and day 180. This observation provides evidence for an age- and breed-related disparity in the expression of *miR-486/sANK1* genes during the postnatal development of porcine skeletal muscles. Similar results have been previously reported in studies comparing Lantang pigs and Landrace pigs [13]. Similar to *miR-486-5p*, *sANK1* demonstrates a significantly elevated expression level in the skeletal muscle of Bama mini-pig compared to Landrace pigs at day 60, day 90, and day 180. Consequently, to comprehend the underlying reasons for the heightened expression of *miR-486-5p/sANK1* in Bama mini-pig relative to Landrace pigs, we conducted cloning and analysis of the promoter region of the porcine *miR-486/sANK1* gene. Our speculation is that the −417 to +153 bp region potentially serves as the core promoter of the porcine sANK1 gene, as mammalian core promoters are typically situated within the range of 100–200 bp upstream of the TSS. The enrichment of guanine clusters and TFBs in the promoter region of a gene has been demonstrated to play a significant role in the regulation of gene expression [14]. SNPs situated within the TFBs of genome regulatory regions have the potential to influence the binding of transcription factors. This, in turn, can lead to the dysregulation of gene transcription, resulting in variations in the level or timing of gene expression and subsequent phenotypic differences [15, 16]. Hence, a comprehensive examination of the transcription factor binding site within the promoters of porcine *miR-486/sANK1* was conducted. The findings unveiled the presence of 18 SNP sites, two deletions, and several transcription factors binding sites, predominantly enriched in muscle tissue-specific transcription factors. Notably, within theseTFBs, fourteen SNPs within the regulatory regions of the *sANK1* gene were identified, resulting in the creation or disruption of putative transcription factor binding motifs. As a result, the capacity of specific muscle-specific transcription factors, namely *MyoD* and *SP1*, to bind to the corresponding sequence is compromised. Consequently, it is hypothesized that the SNPs present in the promoter region of porcine *sANK1* may influence the promoter's functionality, thereby resulting in

distinct expression levels of *miR486/sANK1* in Bama mini-pig and Landrace-pig.

To validate the regulatory SNPs in the promoter region that influence the activity of the Bama mini-pig and Landrace-pig promoters, we conducted experiments to assess the promoter activity derived from these two distinct pig breeds. As anticipated, the promoter of the *sANK1* gene successfully induced the expression of green fluorescent protein (GFP) in C2C12 cells, but not in PK15 cells and PEF cells, indicating its muscle-specific nature. Furthermore, the *sANK1* gene promoter activity in Bama mini-pig was significantly higher than that in Landrace-pig, providing confirmation that ten SNPs within the regulatory regions of the *sANK1* gene may indeed impact the activity of the *sANK1* promoter. It is noteworthy to consider the presence of a nuclear mutation A-G at position −401, specifically related to the TSS, within the Bama mini-pig. This mutation potentially leads to the formation of putative transcription factor binding motifs of *MyoD*, which in turn may play a role in the increased expression of *miR-486/sANK1*.

Genes that possess a shared function, such as participation in a common biological pathway, tend to demonstrate analogous regulatory mechanisms' resulting in the manifestation of co-expressed patterns within their expression profiles [17]. Hence, it can be inferred that the co-expression patterns of *miR-486-5p* and *sANK1* genes, which are subject to temporal variations, are potentially implicated in biologically interconnected processes. The role of *miR-486-5p* in pork quality traits remains unexplored in existing literature. Conversely, the *ANK1* gene emerges as a promising biomarker for assessing meat quality [18]. Recent studies have demonstrated a significant correlation between the expression level of porcine *ANK1* and both pH and IMF in the Basque breed and Large White breed [19, 20], and another report showed that *ANK1* expression was positively correlated with drip loss in the Large White breed [21]. Our previous research has confirmed that the muscle conductivity of Bama mini-pig is significantly lower than that of Landrace pigs, while the intramuscular fat content of Bama mini-pig is significantly higher than that of Landrace pigs [22]. This study reveals a noteworthy upregulation of *miR-486-5p* and *sANK1* in Bama mini-pig, potentially associated with reduced muscle conductivity and increased IMF in this particular breed. Additionally, *sANK1* gene SNPs were found to be linked to various meat quality attributes, such as tenderness, IMF content, pH levels, water-holding capacity, drip loss in pigs, as well as texture scores, juiciness in beef cattle [21, 23, 24]. Therefore, the identification of fourteen SNPs within the regulatory regions of porcine *sANK1* in our study suggests a potential association with variations in meat quality between Bama mini-pig and Landrace-pig. However, additional investigations are required to validate these findings.

## Conclusions

The presented findings in this study provide essential molecular insights and expression patterns of *miR-486-5p/sANK1*, which can be of great significance in enhancing comprehension of the gene's participation in porcine skeletal muscle development and meat quality.

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**Data availability** No datasets were generated or analysed during the current study.

## Declarations

**Ethical approval** The animal study protocol was approved by the Guangxi University (protocol code: GXU2013002; date of approval: 02-2013).

**Competing interests** The authors declare no competing interests.

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