

Article

Molecular Cloning, Structural Analysis and Tissue Expression of *Protein Phosphatase 3 Catalytic Subunit Alpha Isoform (PPP3CA)* Gene in Tianfu Goat Muscle

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Abstract: Calcineurin, a Ca²⁺/calmodulin-dependent protein phosphatase, plays a critical role in controlling skeletal muscle fiber type. However, little information is available concerning the expression of calcineurin in goat. Therefore, *protein phosphatase 3 catalytic subunit alpha isoform (PPP3CA)* gene, also called *calcineurin A α* , was cloned and its expression characterized in Tianfu goat muscle. Real time quantitative polymerase chain reaction (RT-qPCR) analyses revealed that Tianfu goat *PPP3CA* was detected in cardiac muscle, biceps femoris muscle, abdominal muscle, longissimus dors muscle, and soleus muscle. High expression levels were found in biceps femoris muscle, longissimus muscle and abdominal muscle ($p < 0.01$), and low expression levels were seen in cardiac muscle and soleus muscle ($p > 0.05$). In addition, the spatial-temporal mRNA expression levels showed different variation trends in different muscles with the age of the goats. Western blotting further revealed that PPP3CA protein was expressed in the above-mentioned tissues, with the highest level in biceps femoris muscle, and the lowest level in soleus muscle. In this study, we isolated the full-length coding sequence of Tianfu goat *PPP3CA* gene, analyzed its structure, and investigated its expression in different muscle tissues from different age stages. These results provide a foundation for understanding the function of the *PPP3CA* gene in goats.

Keywords: *PPP3CA*; cDNA clone; sequence analysis; structure prediction; expression analysis

1. Introduction

Muscle fiber, significantly affecting the meat quality of livestock, has become a focus research area [1–3]. There are four muscle fiber types, including, slow-oxidative (Type I) muscle fibers, fast oxido-glycolytic (Type IIA) muscle fibers, and fast glycolytic (Type IIX and IIB) muscle fibers [4,5]. One study found that the main way to improve livestock meat quality is meant to raise the proportion of slow-oxidative (Type I) muscle fibers [6].

Calcineurin (CaN), also named protein phosphatase 3 (formerly 2B), is the only serine/threonine protein phosphatase under the control of Ca^{2+} /calmodulin, and plays a critical role in the coupling of Ca^{2+} signals to cellular responses [7]. Ca^{2+} signaling plays a central role in hypertrophic growth of cardiac and skeletal muscle in response to mechanical load and a variety of signals [8]. Therefore, calcineurin plays an important role in muscle differentiation, especially in muscle fiber type conversion. It can differentially regulate genes in oxidative muscle fiber type fast myosin heavy chain conversion, evidently affecting the livestock meat quality [9–13]. On further study of the effects of calcineurin on muscle differentiation, it seems that calcineurin regulates muscle fiber types of muscle growth, depending both on muscle phenotype and stage of myofiber growth, using the calcineurin inhibitor cyclosporin A (CsA) [14,15]. This illustrates that *calcineurin*, is regarded as a candidate gene, that can play a role in muscle fiber differentiation, affecting the meat quality of livestock [16].

Regardless of the source of calcineurin, calcineurin is always a heterodimer of a 58–64 kD catalytic and calmodulin-binding subunit, calcineurin A (CnA), tightly bound to a regulatory 19 kD Ca^{2+} -binding regulatory subunit, calcineurin B (CnB) [17]. CnA can be coded by three different genes, respectively, *PPP3CA* (isoform α), *PPP3CB* (isoform β) and *PPP3CC* (isoform γ), while CnB is encoded by two different genes, respectively, *PPP3R1* (CnB1) and *PPP3R2* (CnB2) [18]. Of all these genes, *PPP3CA* gene is highly expressed in skeletal muscle and takes a part in slow muscle fiber type switching [19]. *PPP3CA* gene, is widely distributed [7,20–23], has also been cloned in human, mouse, pig and cattle, and its encoded-protein is highly conservative [24–27].

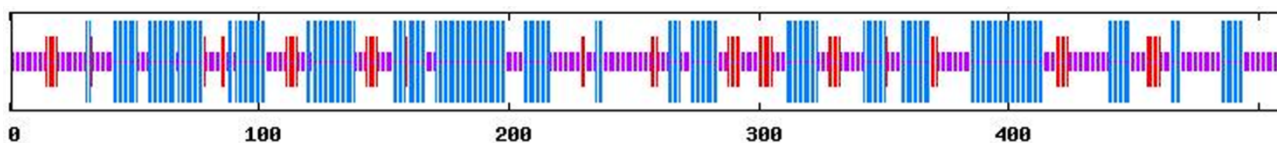
PPP3CA gene has been isolated and its expression analyzed in other mammals already, but there is little to report on caprine. In our study, we cloned Tianfu goat *PPP3CA* gene, analyzed its cDNA sequence and encoded-protein sequence, and examined its expression in different muscle tissues by real time quantitative polymerase chain reaction (RT-qPCR) and western blotting. The results of this study provide a foundation for understanding the function of the *PPP3CA* gene in muscle fiber type conversion.

2. Results and Discussion

2.1. Summary of Characteristics of Tianfu Goat PPP3CA Sequences and Structures

The cDNA nucleotide sequence analysis revealed that the nucleotide sequence represented one open reading frame (ORF), which contained 1536 bp encoded 511 amino acids (Supplementary Information). A BLAST search of NCBI's nucleotide sequence database revealed that the fragment was significantly similar to the *PPP3CA* gene sequences from other mammals. The deduced amino acid sequence of Tianfu goat *PPP3CA* gene, having a molecular weight of 58.0 kDa and pI (isoelectric point) of 5.91, was analyzed with the ProtParam tool. The Tianfu goat *PPP3CA* protein was not a signal peptide, and had no obvious transmembrane domain. There were 33 phosphorylation sites successfully predicted by the neural network in Tianfu goat *PPP3CA* protein. The secondary structure of the *PPP3CA* protein was predicted to be mainly α -helix (43.25%) and random coil (45.79%) (Figure 1). A conserved domain, named PP2Ac, was calculated using the NCBI CD-search and the SMART. The PP2Ac domain, from 56 to 347 AA, was a part of protein phosphatase 2A homologues catalytic domain, a large family of serine phosphatases, which includes PP1, PP2A and PP2B (calcineurin) family members, inferring the conserved domain of PP2Ac, having a similar function with serine phosphatases, and maybe a functional area.

Figure 1. Predicted secondary structure of Tianfu goat *PPP3CA* amino acid (AA) sequence. The blue line represents α -helix, the red line represents extended strand, and the purple line represents random coil.



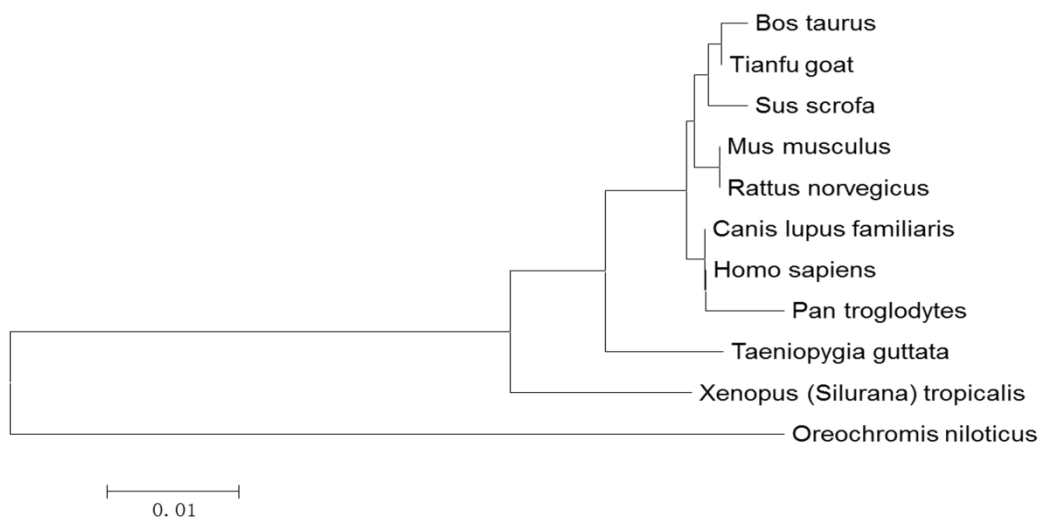
Sequence alignment using DNAMAN V6 software revealed that the coding nucleotide sequence of the Tianfu goat *PPP3CA* gene was 97.1% identical to bovine *PPP3CA*, and 96.7%, 93.9%, 91.2%, 90.6%, 95.6%, 92.7%, 86.8%, 83.2% and 76.2% identical to porcine, dog, mice, rat, human, chimpanzee, finch, frog and tilapia, respectively, and the amino acid sequences were 99.8%, 99.6%, 97.7%, 97.7%, 97.7%, 99.6%, 96.0%, 96.2%, 95.0% and 87.2% identical to bovine, porcine, dog, mice, rat, human, chimpanzee, finch, frog and tilapia, respectively (Figure 2). Sequence alignment revealed that *PPP3CA* proteins from different species were highly conservative.

Amino acid sequences of *PPP3CA* from 11 species were used for the phylogenetic tree constructed with the software MEGA5.10. The results showed that all of the *PPP3CA* proteins from different animals were divided into one subgroup. The highest homology was with cattle, the lowest homology was with tilapia (bony fishes) (Figure 3).

Figure 2. Amino acid sequence alignment of PPP3CA from different species. The blue, pink and blue-green represent the different levels of amino acid sequence similarity of PPP3CA from different species (high, intermediate, and low similarity, respectively).

Bos taurus	MSEFKADPKLSTIDRWKRVVFPFSSHRLTAKEVFDNGKRFVVDLKAHLMEGRLEBSVALRIDEGASILRCEKNLLDIDAFVTVCGDIHQGFDDLMK	100
Canis lupus familiaris	MSEFKADPKLSTIDRWKRVVFPFSSHRLTAKEVFDNGKRFVVDLKAHLMEGRLEBSVALRIDEGASILRCEKNLLDIDAFVTVCGDIHQGFDDLMK	100
Homo sapiens	MSEFKADPKLSTIDRWKRVVFPFSSHRLTAKEVFDNGKRFVVDLKAHLMEGRLEBSVALRIDEGASILRCEKNLLDIDAFVTVCGDIHQGFDDLMK	100
Mus musculus	MSEFKADPKLSTIDRWKRVVFPFSSHRLTAKEVFDNGKRFVVDLKAHLMEGRLEBSVALRIDEGASILRCEKNLLDIDAFVTVCGDIHQGFDDLMK	100
Oreochromis niloticus	MVSANSLSCREPIFHPQTVVFPFSSHRLTAKEVFDNGKRFVVDLKAHLMEGRLEBSVALRIDEGASILRCEKNLLDIDAFVTVCGDIHQGFDDLMK	100
Pan troglodytes	MSEFKADPKLSTIDRWKRVVFPFSSHRLTAKEVFDNGKRFVVDLKAHLMEGRLEBSVALRIDEGASILRCEKNLLDIDAFVTVCGDIHQGFDDLMK	100
Rattus norvegicus	MSEFKADPKLSTIDRWKRVVFPFSSHRLTAKEVFDNGKRFVVDLKAHLMEGRLEBSVALRIDEGASILRCEKNLLDIDAFVTVCGDIHQGFDDLMK	100
Sus scrofa	MSEFKADPKLSTIDRWKRVVFPFSSHRLTAKEVFDNGKRFVVDLKAHLMEGRLEBSVALRIDEGASILRCEKNLLDIDAFVTVCGDIHQGFDDLMK	100
Taeniopygia guttata	MSEHTVDFALSTIDRWKRVVFPFSSHRLTAKEVFDNGKRFVVDLKAHLMEGRLEBSVALRIDEGASILRCEKNLLDIDAFVTVCGDIHQGFDDLMK	100
Tianfu goat	MSEFKADPKLSTIDRWKRVVFPFSSHRLTAKEVFDNGKRFVVDLKAHLMEGRLEBSVALRIDEGASILRCEKNLLDIDAFVTVCGDIHQGFDDLMK	100
Xenopus Silurana tropicalis	MSEHRANEAKLSTIDRWKRVVFPFSSHRLTAKEVFDNGKRFVVDLKAHLMEGRLEBSVALRIDEGASILRCEKNLLDIDAFVTVCGDIHQGFDDLMK	100
Consensus	m v vfpfsshrlltakevfdngkrfvvd lkahl megrlebsvalr idegasilr ceknll didafv tvcgdihqgffddmk	
Bos taurus	LFEVGGSEPAITRYLFLGDYVDRGYFSIECVLYLWALKILYPTLFLLRGNHECRHLEYFTFKQCEKRIKYSSEVYDAMDADFCLPLAALMNQGFCLVHG	200
Canis lupus familiaris	LFEVGGSEPAITRYLFLGDYVDRGYFSIECVLYLWALKILYPTLFLLRGNHECRHLEYFTFKQCEKRIKYSSEVYDAMDADFCLPLAALMNQGFCLVHG	200
Homo sapiens	LFEVGGSEPAITRYLFLGDYVDRGYFSIECVLYLWALKILYPTLFLLRGNHECRHLEYFTFKQCEKRIKYSSEVYDAMDADFCLPLAALMNQGFCLVHG	200
Mus musculus	LFEVGGSEPAITRYLFLGDYVDRGYFSIECVLYLWALKILYPTLFLLRGNHECRHLEYFTFKQCEKRIKYSSEVYDAMDADFCLPLAALMNQGFCLVHG	200
Oreochromis niloticus	LFEVGGSEPAITRYLFLGDYVDRGYFSIECVLYLWALKILYPTLFLLRGNHECRHLEYFTFKQCEKRIKYSSEVYDAMDADFCLPLAALMNQGFCLVHG	200
Pan troglodytes	LFEVGGSEPAITRYLFLGDYVDRGYFSIECVLYLWALKILYPTLFLLRGNHECRHLEYFTFKQCEKRIKYSSEVYDAMDADFCLPLAALMNQGFCLVHG	200
Rattus norvegicus	LFEVGGSEPAITRYLFLGDYVDRGYFSIECVLYLWALKILYPTLFLLRGNHECRHLEYFTFKQCEKRIKYSSEVYDAMDADFCLPLAALMNQGFCLVHG	200
Sus scrofa	LFEVGGSEPAITRYLFLGDYVDRGYFSIECVLYLWALKILYPTLFLLRGNHECRHLEYFTFKQCEKRIKYSSEVYDAMDADFCLPLAALMNQGFCLVHG	200
Taeniopygia guttata	LFEVGGSEPAITRYLFLGDYVDRGYFSIECVLYLWALKILYPTLFLLRGNHECRHLEYFTFKQCEKRIKYSSEVYDAMDADFCLPLAALMNQGFCLVHG	200
Tianfu goat	LFEVGGSEPAITRYLFLGDYVDRGYFSIECVLYLWALKILYPTLFLLRGNHECRHLEYFTFKQCEKRIKYSSEVYDAMDADFCLPLAALMNQGFCLVHG	200
Xenopus Silurana tropicalis	LFEVGGSEPAITRYLFLGDYVDRGYFSIECVLYLWALKILYPTLFLLRGNHECRHLEYFTFKQCEKRIKYSSEVYDAMDADFCLPLAALMNQGFCLVHG	200
Consensus	lfevggsepa itrylflgd yvdrgyfsiecvlylwalkilyptlfl lrgnhechrhleyftfkqceckikyse vydamc dmfclplaalmnqg fclvhg	
Bos taurus	GLSFEINTLDDIRKLRDFKEPPAYGPMCDLWSDPLEDGNKQCEHFTHTVRCGSFHY...SYPAVCFIQHNNLLSIRAEHAQDAGYRMVRSQT	296
Canis lupus familiaris	GLSFEINTLDDIRKLRDFKEPPAYGPMCDLWSDPLEDGNKQCEHFTHTVRCGSFHY...SYPAVCFIQHNNLLSIRAEHAQDAGYRMVRSQT	296
Homo sapiens	GLSFEINTLDDIRKLRDFKEPPAYGPMCDLWSDPLEDGNKQCEHFTHTVRCGSFHY...SYPAVCFIQHNNLLSIRAEHAQDAGYRMVRSQT	296
Mus musculus	GLSFEINTLDDIRKLRDFKEPPAYGPMCDLWSDPLEDGNKQCEHFTHTVRCGSFHY...SYPAVCFIQHNNLLSIRAEHAQDAGYRMVRSQT	296
Oreochromis niloticus	GLSFEINTLDDIRKLRDFKEPPAYGPMCDLWSDPLEDGNKQCEHFTHTVRCGSFHY...SYPAVCFIQHNNLLSIRAEHAQDAGYRMVRSQT	296
Pan troglodytes	GLSFEINTLDDIRKLRDFKEPPAYGPMCDLWSDPLEDGNKQCEHFTHTVRCGSFHY...SYPAVCFIQHNNLLSIRAEHAQDAGYRMVRSQT	300
Rattus norvegicus	GLSFEINTLDDIRKLRDFKEPPAYGPMCDLWSDPLEDGNKQCEHFTHTVRCGSFHY...SYPAVCFIQHNNLLSIRAEHAQDAGYRMVRSQT	296
Sus scrofa	GLSFEINTLDDIRKLRDFKEPPAYGPMCDLWSDPLEDGNKQCEHFTHTVRCGSFHY...SYPAVCFIQHNNLLSIRAEHAQDAGYRMVRSQT	296
Taeniopygia guttata	GLSFEINTLDDIRKLRDFKEPPAYGPMCDLWSDPLEDGNKQCEHFTHTVRCGSFHY...SYPAVCFIQHNNLLSIRAEHAQDAGYRMVRSQT	296
Tianfu goat	GLSFEINTLDDIRKLRDFKEPPAYGPMCDLWSDPLEDGNKQCEHFTHTVRCGSFHY...SYPAVCFIQHNNLLSIRAEHAQDAGYRMVRSQT	296
Xenopus Silurana tropicalis	GLSFEINTLDDIRKLRDFKEPPAYGPMCDLWSDPLEDGNKQCEHFTHTVRCGSFHY...SYPAVCFIQHNNLLSIRAEHAQDAGYRMVRSQT	296
Consensus	glsfei intl ddirklr dfkeppaygpmcd lwsdp ledgnk qehfthntvrcgsfhy f sypavc fiqhnnlls iraehaqd agyrmvrsqt	
Bos taurus	TGFPSLITIFSAPNYLDVYNNKAAVLYENNVNMRQFCNSPHPYLWPNFMDVFTWSLFPVGEKVTMLVVLVICSDELGSEEDGFGATAARKEVI	396
Canis lupus familiaris	TGFPSLITIFSAPNYLDVYNNKAAVLYENNVNMRQFCNSPHPYLWPNFMDVFTWSLFPVGEKVTMLVVLVICSDELGSEEDGFGATAARKEVI	396
Homo sapiens	TGFPSLITIFSAPNYLDVYNNKAAVLYENNVNMRQFCNSPHPYLWPNFMDVFTWSLFPVGEKVTMLVVLVICSDELGSEEDGFGATAARKEVI	396
Mus musculus	TGFPSLITIFSAPNYLDVYNNKAAVLYENNVNMRQFCNSPHPYLWPNFMDVFTWSLFPVGEKVTMLVVLVICSDELGSEEDGFGATAARKEVI	396
Oreochromis niloticus	TGFPSLITIFSAPNYLDVYNNKAAVLYENNVNMRQFCNSPHPYLWPNFMDVFTWSLFPVGEKVTMLVVLVICSDELGSEEDGFGATAARKEVI	395
Pan troglodytes	TGFPSLITIFSAPNYLDVYNNKAAVLYENNVNMRQFCNSPHPYLWPNFMDVFTWSLFPVGEKVTMLVVLVICSDELGSEEDGFGATAARKEVI	400
Rattus norvegicus	TGFPSLITIFSAPNYLDVYNNKAAVLYENNVNMRQFCNSPHPYLWPNFMDVFTWSLFPVGEKVTMLVVLVICSDELGSEEDGFGATAARKEVI	396
Sus scrofa	TGFPSLITIFSAPNYLDVYNNKAAVLYENNVNMRQFCNSPHPYLWPNFMDVFTWSLFPVGEKVTMLVVLVICSDELGSEEDGFGATAARKEVI	396
Taeniopygia guttata	TGFPSLITIFSAPNYLDVYNNKAAVLYENNVNMRQFCNSPHPYLWPNFMDVFTWSLFPVGEKVTMLVVLVICSDELGSEEDGFGATAARKEVI	396
Tianfu goat	TGFPSLITIFSAPNYLDVYNNKAAVLYENNVNMRQFCNSPHPYLWPNFMDVFTWSLFPVGEKVTMLVVLVICSDELGSEEDGFGATAARKEVI	396
Xenopus Silurana tropicalis	TGFPSLITIFSAPNYLDVYNNKAAVLYENNVNMRQFCNSPHPYLWPNFMDVFTWSLFPVGEKVTMLVVLVICSDELGSEEDGFGATAARKEVI	396
Consensus	tgfpsl itifsapny ldvynnkaavlyennvnmr qfcnsphpyl wpnfmdvftwslfpvgekvtmlv vlics del gse edgfgata arkevi	
Bos taurus	RNKIRAIQKMARVSVLREESESVLTLRGLTPTGMLPSGVLGGKQTLQSAITVEAIEADBAIKGFSQHKITSFEEAKGLDRINERMPFRDAMPSDAL	486
Canis lupus familiaris	RNKIRAIQKMARVSVLREESESVLTLRGLTPTGMLPSGVLGGKQTLQSAITVEAIEADBAIKGFSQHKITSFEEAKGLDRINERMPFRDAMPSDAL	496
Homo sapiens	RNKIRAIQKMARVSVLREESESVLTLRGLTPTGMLPSGVLGGKQTLQSAITVEAIEADBAIKGFSQHKITSFEEAKGLDRINERMPFRDAMPSDAL	486
Mus musculus	RNKIRAIQKMARVSVLREESESVLTLRGLTPTGMLPSGVLGGKQTLQSAITVEAIEADBAIKGFSQHKITSFEEAKGLDRINERMPFRDAMPSDAL	496
Oreochromis niloticus	RNKIRAIQKMARVSVLREESESVLTLRGLTPTGMLPSGVLGGKQTLQSAITVEAIEADBAIKGFSQHKITSFEEAKGLDRINERMPFRDAMPSDAL	495
Pan troglodytes	RNKIRAIQKMARVSVLREESESVLTLRGLTPTGMLPSGVLGGKQTLQSAITVEAIEADBAIKGFSQHKITSFEEAKGLDRINERMPFRDAMPSDAL	500
Rattus norvegicus	RNKIRAIQKMARVSVLREESESVLTLRGLTPTGMLPSGVLGGKQTLQSAITVEAIEADBAIKGFSQHKITSFEEAKGLDRINERMPFRDAMPSDAL	496
Sus scrofa	RNKIRAIQKMARVSVLREESESVLTLRGLTPTGMLPSGVLGGKQTLQSAITVEAIEADBAIKGFSQHKITSFEEAKGLDRINERMPFRDAMPSDAL	486
Taeniopygia guttata	RNKIRAIQKMARVSVLREESESVLTLRGLTPTGMLPSGVLGGKQTLQSAITVEAIEADBAIKGFSQHKITSFEEAKGLDRINERMPFRDAMPSDAL	495
Tianfu goat	RNKIRAIQKMARVSVLREESESVLTLRGLTPTGMLPSGVLGGKQTLQSAITVEAIEADBAIKGFSQHKITSFEEAKGLDRINERMPFRDAMPSDAL	486
Xenopus Silurana tropicalis	RNKIRAIQKMARVSVLREESESVLTLRGLTPTGMLPSGVLGGKQTLQSAITVEAIEADBAIKGFSQHKITSFEEAKGLDRINERMPFRDAMPSDAL	496
Consensus	rnk irai qkmarv svl ree sesv lt rgl tpt gml ps gvl gsk qlq s ai tvea iead ba ik gfs qhk its fee a kgl dr in er mp frd am ps da l	
Bos taurus	NSNKAISSETNGTDSGSS.NI	510
Canis lupus familiaris	NSNKAISSETNGTDSGSS.NI	520
Homo sapiens	NSNKAISSETNGTDSGSS.NI	510
Mus musculus	NSNKAISSETNGTDSGSS.NI	520
Oreochromis niloticus	NSNKAISSETNGTDSGSS.NI	521
Pan troglodytes	NSNKAISSETNGTDSGSS.NI	524
Rattus norvegicus	NSNKAISSETNGTDSGSS.NI	520
Sus scrofa	NSNKAISSETNGTDSGSS.NI	510
Taeniopygia guttata	NSNKAISSETNGTDSGSS.NI	519
Tianfu goat	NSNKAISSETNGTDSGSS.NI	510
Xenopus Silurana tropicalis	NSNKAISSETNGTDSGSS.NI	520
Consensus	ns nka is setngt ds gss ni	

Figure 3. Phylogenetic tree of the amino acid sequences of PPP3CA. The tree was constructed using the Neighbor Joining method in the MEGA 5.10 software. All above sequences are from the NCBI data base (*Bos mutus*: NP_777212.1; *Sus scrofa*: NP_999293.1; *Homo sapiens*: NP_000935.1; *Pan troglodytes*: XP_001168163.1; *Canis lupus familiaris*: NP_001184025.1; *Mus musculus*: NP_058737.1; *Rattus norvegicus*: NP_058737.1; *Taeniopygia guttata*: XP_002193146.2; *Oreochromis niloticus*: XP_004554421.1; and *Xenopus (Silurana) tropicalis*: NP_001119980.1).



A SWISS-MODEL server was used to construct a 3D structural model of Tianfu goat PPP3CA amino acid sequence (between amino acids (AA) 1–352). The homology modeling revealed that this segment was similar to that of the ZN505 in the Protein Data Bank (ZN505: 1tcoA: 21–372 AA) (Figure 4). In Figure 5, there is no difference between PPP3CA (1–352 AA) protein domain and ZN505-1tcoA (21–372 AA) domain. The 3D structural model of Tianfu goat may provide a basis for further studying the relationship between structure and function of PPP3CA protein in goats.

Figure 4. The 3D structural model of Tianfu goat PPP3CA (1–352 AA) based on homology modeling.



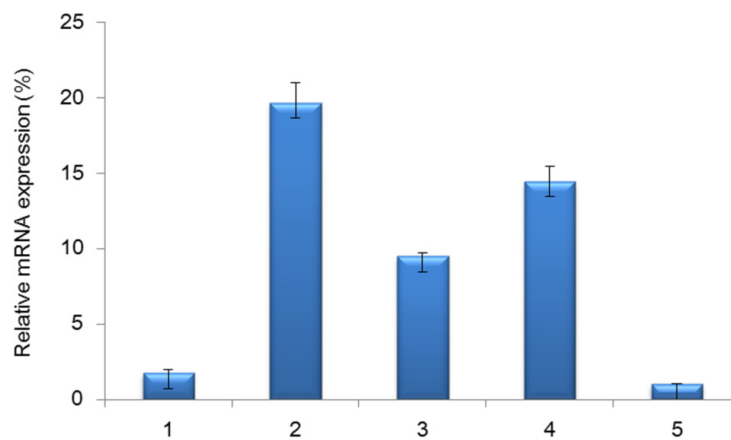
Figure 5. Alignment of the Tianfu goat PPP3CA and ZN505 amino acid sequences. TARGET represents Tianfu goat PPP3CA (1–352 AA), 1tcoA represents ZN505 (21–372 AA); s indicates the residues that form the “bend”; and h indicates the residues that form the “ α -helix”.

TARGET	1	VFPFSSHRLTAKEVFDNDGKPRVDILKALHMKEGRLLEETVALRIITEG
1tcoA	21	vpfppshr ltakevfdnd gkprvdilka hlmkegrlee tvalriiteg
TARGET		hhhh hhhhhh hhh h hhhhhhhhhh
1tcoA		hhhh hhhhhh hhh h hhhhhhhhhh
TARGET	49	ASILRQEKQLLDIDAPVTVC GDINGQFFDL MCLFEVGGSP ANTRYLFLGD
1tcoA	69	asilrqeknl ldidapvtvc gdihgqffdl mclfevggsp antrylflgd
TARGET		hhhhh sss ssss hhhh hhhhhh sssss
1tcoA		hhhhh sss ssss hhhh hhhhhh sssss
TARGET	99	YVDRGYFSIE CVLYLWALKI LYPKTLFLLR GNHECRHLTE YFTFKQECKI
1tcoA	119	yvdrgyfsie cvlylwalki lypktlfllr gnhechrhlte yftfkqecki
TARGET		hhh hhhhhhhhhh h sssss hhhh hhhhhh
1tcoA		hhh hhhhhhhhhh h sssss hhhh hhhhhh
TARGET	149	KYSERVYDAC MDAFDCLPLA ALMNQQFLCV HGGLSPEINT LDDIRKLDRF
1tcoA	169	kyservydac mdafdclpla almnqqflcv hgglspeint lddirkldr f
TARGET		hhhhhhh hhhh s ssss ssss hhhh
1tcoA		hhhhhhh hhhh s ssss ssss hhhh
TARGET	199	KEPPAYGPMC DILWSDPLED FGNEKTQEHF THNTVRCGSY FYSYPVACEF
1tcoA	219	keppaygpmc dilwsdpled fgnektqehf thntvrgcsy fysypavcef
TARGET		hhh hhh sss ss sssss ssss ssshhhhhhh
1tcoA		hhh hhhh ss sssss ssss ssshhhhhhh
TARGET	249	LQHNNLSIL RAHEAQDAGY RMYRKSQITG FPSLITIFSA PNYLDVYNNK
1tcoA	269	lqhnnlsil raheaqdagy rmyrksqitg fpsliti fsa pnyldvynnk
TARGET		hhhh sss s ss ssssss ssssssss
1tcoA		hhhh sss s ss ssssss ssssssss
TARGET	299	AAVLKYENNV MNIRQFNCSP HPYWLPNFMD VFTWSLPFVG EKVTMLVNV
1tcoA	319	aavlkyyennv mnirqfncsp hpywlpnfmv vftwslp fvg ekvtmlv nv
TARGET		sssssss s ssssss hhhhhhhhhh hhhhhhhhhh
1tcoA		sssssss s ssssss hhhhhhhhhh hhhhhhhhhh
TARGET	349	LNIC
1tcoA	369	lnic-
TARGET		h
1tcoA		h

2.2. Analysis of mRNA Expression of Tianfu Goat PPP3CA in Different Muscle

RT-qPCR was conducted to determine the spatial-temporal mRNA expression of *PPP3CA* gene in Tianfu goat muscle tissues, employing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous control in each sample. The RT-qPCR results showed that Tianfu goat *PPP3CA* mRNA existed in cardiac muscle, biceps femoris muscle, abdominal muscle, longissimus dors muscle and soleus muscle at 150 days old (Figure 6). Particularly, high expression levels of *PPP3CA* were found in biceps femoris muscle, longissimus dors muscle and abdominal muscle ($p < 0.01$), and low expression levels were observed in cardiac muscle and soleus muscle ($p > 0.05$). This result is consistent with previous research in pig (Figure 6) [26]. In former studies, Pette *et al.*, showed that the muscle fiber type changed after birth [28]. That is to say, proportions of fast and slow muscle fiber types from different muscle tissues were different. It stands to reason that the *PPP3CA* gene was differently expressed in different muscle tissues with different proportions of fast and slow muscle fibers, suggesting the *PPP3CA* gene is the key to muscle fiber growth and development [22,26].

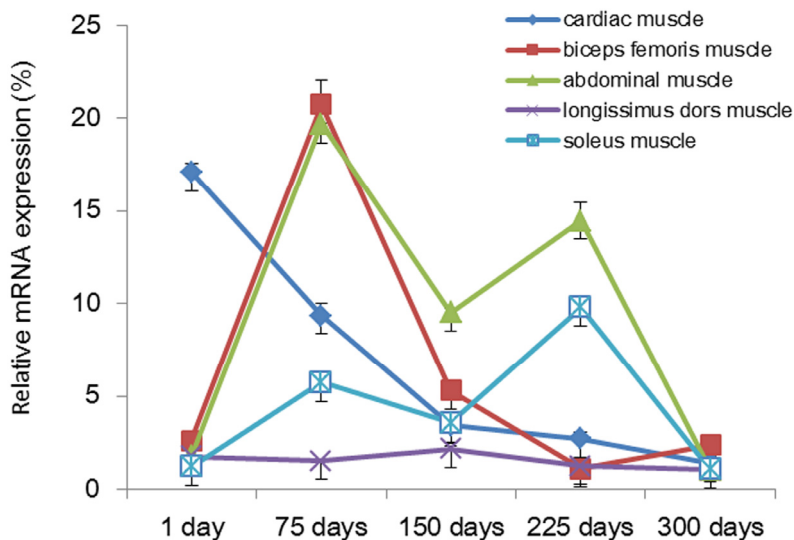
Figure 6. Spatial mRNA expression profile of Tianfu goat *PPP3CA* gene. The samples 1–5 represent cardiac muscle, biceps femoris muscle, abdominal muscle, longissimus dors muscle and soleus muscle, respectively. Bars represent the mean \pm SD ($n = 8$).



In addition, the *PPP3CA* spatial-temporal mRNA expression levels in different muscles showed different variation trends in five muscle tissues with the age of Tianfu goats. During cardiac muscle development, gene expression of *PPP3CA* was decreased gradually from the day 1 to the day 300; the highest expression level was seen on the day 1 and the lowest expression level on the day 300 (Figure 7). In biceps femoris muscle, gene expression of *PPP3CA* was first increased from the day 1 to the day 75, then decreased to the day 225 and finally increased to the day 300; the highest expression was seen on the day 75 and the lowest on the day 225 (Figure 7). In abdominal muscle, gene expression of *PPP3CA* was first increased from the day 1 to the day 75, decreased to the day 150, after which it increased to the day 225, and finally decreased to the day 300; the highest expression was observed on the day 75 and the lowest on the day 300 (Figure 7). This contrasts with *PPP3CA* gene expression in soleus muscle, which was highest on the day 225 and lowest on the day 300. In longissimus dors muscle, gene expression of *PPP3CA* was first decreased from the day 1 to the day 75, then increased to the day 150 and finally decreased to the day 300; the highest expression was seen on the day 150 and the lowest on the day 300 (Figure 7). Among these trends, the change curves of gene expression of *PPP3CA* appeared to have remarkable fluctuation over the period from the day 1 to the day 300 in cardiac muscle, biceps femoris muscle, abdominal muscle, and soleus muscle (Figure 7). However, during that time, there was a slight change of *PPP3CA* gene expression in longissimus dors muscle (Figure 7).

McCoard *et al.*, previously found that the proportions of slow muscle fibers grew steadily in different muscles of lamb [29]. During muscle growth and development, the mRNA expression of *PPP3CA* gene showed different variation trends in five muscle tissues with different proportions of slow muscle fiber (Figure 7), implying *PPP3CA* could take a major part in slow muscle fiber differentiation [23]. However, a special trend, a slight variation trend of *PPP3CA* gene expression, was found in longissimus dors muscle (Figure 7). After birth, muscle fibers of longissimus dors muscle were found primarily to have more fast muscle fibers than their counterparts in other muscle tissues, signifying they were differentiated into fast muscle fibers in longissimus dors muscle [1,30]. Consequently, gene expression of *PPP3CA* slowed varying transformation of fast muscle fibers in longissimus dors muscle from different age stages of Tianfu goats. These results further confirmed that *PPP3CA* may serve a function in controlling slow muscle fiber differentiation [19].

Figure 7. Temporal mRNA expression profiles of *PPP3CA* during different muscle development in Tianfu goat. Bars represent the mean \pm SD ($n = 8$).

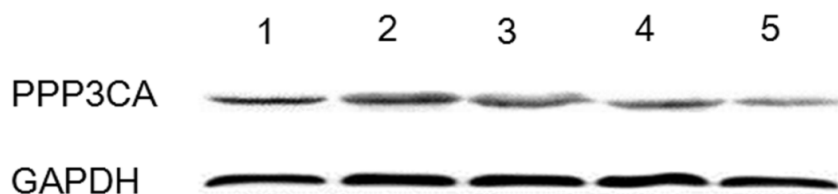


2.3. Western Blotting Analysis of Protein Expression of Tianfu Goat *PPP3CA*

Western blotting results showed different expression levels of *PPP3CA* protein in cardiac muscle, biceps femoris muscle, abdominal muscle, longissimus dors muscle, and soleus muscle at 150 days old. Using GAPDH as the reference protein in each sample, *PPP3CA* protein was expressed in the above-mentioned muscle tissues, with the highest level in biceps femoris muscle and the lowest level in soleus muscle (Figure 8).

During the muscle fiber development and differentiation, the slow muscle fibers grew steadily in lamb muscles, but with the exception of soleus muscle [29,31]. This means that the slow muscle fiber proportions of soleus muscle were not significantly changed after birth [31]. *PPP3CA* came into play a role in slow muscle fiber differentiation, the more the gene expression of *PPP3CA*, the stronger its effects in slow muscle fiber differentiation [9,10,19]. Thereby, *PPP3CA* protein was expressed at the lowest level in soleus muscle [19]. The results of western blotting further confirmed that *PPP3CA* protein was differently expressed in different muscle tissues with different proportions of muscle fibers. This threatens *PPP3CA* gene required for the key processes of myocyte differentiation and conversion to the slow muscle fiber type [7,12,19].

Figure 8. Western blotting of Tianfu goat *PPP3CA* protein levels in five muscle tissues. The samples 1–5 represent cardiac muscle, biceps femoris muscle, abdominal muscle, longissimus dors muscle and soleus muscle, respectively, ($n = 8$).



3. Experimental Section

3.1. Animals and Sample Collection

All experimental procedures were approved by Sichuan Agricultural University Animal Care and Use Committee, Sichuan Agricultural University, Sichuan, China under permit No. DKY-B20100805. All of the Tianfu goats were bred under the same standard conditions and slaughtered on the 1st, 75th, 150th, 225th, and 300th day after birth ($n = 8$ per group). The five muscle samples, including cardiac muscle, biceps femoris muscle, abdominal muscle, longissimus dors muscle and soleus muscle, were harvested, frozen in liquid nitrogen, and stored at $-80\text{ }^{\circ}\text{C}$ until total RNA and protein extraction.

3.2. Total RNA Isolation and Synthesis of cDNA

According to the manufacturer protocol's, total RNA was extracted from five muscle tissues (cardiac muscle, biceps femoris muscle, abdominal muscle, longissimus dors muscle, and soleus muscle) using the RNAiso Plus (TaKaRa, Dalian, China). Total RNA was detected by 1% agarose gel electrophoresis with $10\times$ loading buffer (Tiangen, Beijing, China), and then preserved at $-80\text{ }^{\circ}\text{C}$. According to the manufacturer's protocol, the first strand cDNA was compounded using a PrimeScriptTM RT Reagent kit (TaKaRa). First strand cDNA was obtained and preserved at $-20\text{ }^{\circ}\text{C}$ a freezer.

3.3. Cloning of the Tianfu Goat PPP3CA Gene

To clone the cDNA sequence of Tianfu goat *PPP3CA* gene, the RNAs were extracted from the longissimus dorsi muscle of Tianfu goat with the following cDNA library construction. A pair of primers, 5'-ATGTCCGAGCCCAAGGCAATT-3' and 5'-AGCAGCAATATCCAGTGA-3', which was designed by the Primer premier 5.0 software (PREMIER Biosoft International, Palo Alto, Canada) and based on the bovine *PPP3CA* gene (GenBank No. NM_174787.2) [32] was prepared for PCR. PCRs were run under the following cycling conditions: $95\text{ }^{\circ}\text{C}$ for 5 min, followed by 40 cycles of $95\text{ }^{\circ}\text{C}$ for 30 s; $61.4\text{ }^{\circ}\text{C}$ for 30 s; $72\text{ }^{\circ}\text{C}$ for 90 s, and a final extension at $72\text{ }^{\circ}\text{C}$ for 10 min. The products of PCRs were detected by 1.5% agarose gel electrophoresis, and then the PCR products were obtained. An E.Z.N.A Gel Extraction Kit (Omega BioTek, Doraville, GA, USA) was used to extract the PCR fragments from the gel, and then ligated into pMD19-T vector (TaKaRa) at $4\text{ }^{\circ}\text{C}$ overnight. Finally, the cloned products were sequenced by LiuHe HuaDa Biotechnology Co. Ltd. (Beijing, China).

3.4. Sequence Analysis

Sequence analysis of the predicted PPP3CA protein of Tianfu goat translated used ExPaSy (<http://www.expasy.org>). The molecular weight and pI (isoelectric point) were divided by ProtParam tool (<http://web.expasy.org/protparam/>). The NetPhos 2.0 server (<http://www.cbs.dtu.dk/services/NetPhos/>) was used to produce neural network predictions for phosphorylation sites in Tianfu goat PPP3CA protein, while SignalP 4.1 server (<http://www.cbs.dtu.dk/services/SignalP/>) was employed to dope out location of signal peptide cleavage sites in PPP3CA amino acid sequence. The transmembrane helices were calculated to preforecast by TMHMM 2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>). The putative conserved

domain of PPP3CA protein was calculated by NCBI Batch Web CD-search tool (<http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>) and SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1) online tool. The secondary structure of deduced amino acid sequence was predicted by NPSA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/%20npsa_hnn.html). The coding nucleotide and protein sequences of PPP3CA from related different species were aligned with the Tianfu goat sequences using the DNAMAN V6 software (Lynnon Biosoft, Los Angeles, CA, USA). Amino acid sequences of PPP3CA protein from 11 species were used for the phylogenetic tree constructed by the MEGA5.10 program. The SWISS-MODEL server (<http://www.expasy.org/swissmod/SWISSMODEL.html>) was used to model the Tianfu goat PPP3CA protein 3D conformation.

3.5. RT-qPCR Analysis of PPP3CA Gene mRNA Expression

Total RNAs of five muscle tissues were extracted. RT-qPCR was used to analyze the relative mRNA expression of *PPP3CA* gene in different muscle tissues of Tianfu goat at 150 days old. Analysis of spatial-temporal expression patterns of *PPP3CA* gene used the housekeeping gene, *GAPDH* gene as an internal control. The primers are shown in Table 1. The 25 μ L reaction system of RT-qPCR was: 12.5 μ L SYBR premix Ex TAq™ (TaKaRa), 1 μ L Forward primer (4.19 nmol per OD), 1 μ L Reverse primer (4.37 nmol per OD), 2 μ L cDNA of each muscle tissue, 8.5 μ L RNase-free H₂O (Tiangen). RT-qPCR program initially started with: 95 °C for 10 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s.

Table 1. Primer pairs used for analysis of the gene expression of Tianfu goat *PPP3CA*.

Primer	Sequence fragment	Product length (bp)	Application
PPP3CA-F	GAACCACCTGCTTATGGACCTAT	244	Expression
PPP3CA-R	AAGGGAAGCCTGTTGTTTGG	244	Expression
GAPDH-F	GTCACCAACTGGGACGACA	118	RT-qPCR
GAPDH-R	AGGCGTACAGGGACAGCA	118	RT-qPCR

3.6. Western Blotting Analyses of PPP3CA Protein Expression

Total proteins of five muscle tissues were extracted by using a Total Protein Extraction Kit (Sangon Biotech, Shanghai, China), according to the manufacturer's protocol, and then the muscle proteins were preserved at -80 °C in a freezer. Western blotting was used to analyze the relative protein expression of PPP3CA in muscle tissues of Tianfu goat at 150 days old. The determination of the protein concentration used BCA protein assay kit (Beyotime, Shanghai, China) with bovine serum albumin (BSA) as a standard. GAPDH protein was selected as an internal control. Polyclonal antibodies to the calcineurin protein (Abcam, Cambridge, UK) were used at 1/500 dilution and a monoclonal antibody to the GAPDH protein (BiYunTian, Shanghai, China) was at a 1/1000 dilution. Equal amounts of protein (12 μ g per lane) were resolved on 12% SDS-polyacrylamide gels, and then as detailed previously in [33], the intensity of the signal was used to analyze the relative amount of PPP3CA protein in Tianfu goat muscle tissues extracts.

3.7. Statistical Analysis

All data are expressed as the means \pm SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test through the SPSS 13.0 statistical software package (SPSS Inc., Chicago, IL, USA). The threshold of significance was defined as $p < 0.05$.

4. Conclusions

In our study, we isolated the Tianfu goat *PPP3CA* gene and used various bioinformatics software to analyze its nucleotide and protein sequences. We predicted a 3D structure of PPP3CA protein (1–352 AA) and analyzed the differences of spatial-temporal mRNA expression in different muscle tissues from five age stages of Tianfu goats. Additionally, western blotting was used to analyze the expression of PPP3CA protein in five muscle tissues of Tianfu goats. The first evidence showed that *PPP3CA* gene is expressed in goat muscle tissues and has an expression pattern similar to porcine, suggesting that a similar function of *PPP3CA* in both goats and pigs. This information obtained provides an important theoretical basis for further research into the function of *PPP3CA*.

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Author Contributions

L.W. and J.M. designed the experiment under the supervision of G.X.; L.W., J.M. and N.W. performed the experiments and analyzed the data with the advice of G.X. and D.W.; L.W. and J.M. discussed the results; G.X. and D.W. gave conceptual advice; W.L. wrote the manuscript; all authors commented on the manuscript at all stages.

Conflicts of Interest

The authors declare no conflict of interest.

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