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Variation in the ovine WFIKKN2 gene

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ABSTRACT

WFIKKN2 may play a role in the regulation of muscle growth and development through its interaction with growth and differentiation factor 8 (GDF8) and growth and differentiation factor 11 (GDF11), but to date research into the function of the protein has been focused on mice, even though the WFIKKN2 gene (*WFIKKN2*) was first identified in humans in 2001.

In this study two regions (intron 1 and the 3′ UTR) of ovine *WFIKKN2* were investigated, using Polymerase Chain Reaction-Single Stranded Conformational Polymorphism (PCR-SSCP). Two different PCR-SSCP patterns, representing two unique DNA sequences (designated *a* and *b*) were detected in a 399-bp amplicon derived from the 3′ UTR, with sequence analysis revealing one single nucleotide polymorphism (SNP). In a 421-bp amplicon from intron 1, five different PCR-SSCP patterns (designated *A*–*E*) were observed and twelve SNPs were detected. Either one or two different sequences were detected in individual sheep and all the sequences identified shared homology with the *WFIKKN2* sequences from cattle and other animal species, suggesting that these sequences represent variants of the ovine WFIKKN2 gene. In intron 1 of 487 sheep from eight breeds, variants *B* and *C* were the most common, followed by *A*, *D* and *E*. These results indicate that ovine *WFIKKN2* is polymorphic and suggest that further analysis is required to see if variation in the gene is associated with variation in growth and muscle traits in sheep.

1. Introduction

WFIKKN2 (also called growth and differentiation factor associated serum protein-1, GASP1) is a large extracellular multi-domain protein that contains a whey acidic protein (WAP)-domain, a follistatin-domain, an immunoglobulin-domain, two Kunitz-type protease inhibitor-domains and a NTR-domain (Trexler et al., 2001). WFIKKN2 was first identified in humans in 2001 (Trexler et al., 2001), but many aspects of its biology in human and livestock species are not understood.

In vitro experiments reveal that WFIKKN2 is a potent inhibitor of two TGF beta family members that are implicated in musculoskeletal development: GDF8 (growth and differentiation factor 8, also known as myostatin) and GDF11 (growth and differentiation factor 11, also known as bone morphogenetic protein 11) (Hill et al., 2003; Kondas et al., 2008). Specifically, 3 nM and 50 nM WFIKKN2 cause 50% and 90% inhibitions of GDF8 and GDF11 activities respectively, using a SPR (surface plasmon resonance) assay in a solution–competition format (Szlama et al., 2010).

GDF8 acts primarily as a negative regulator of muscle development in mammals. Deletions or mutations in the GDF8 gene can cause an increase in skeletal muscle mass as a result of both muscle hypertrophy and hyperplasia in a number of animal species including sheep (Clop et al., 2006), cattle (Gill et al., 2009; Kambadur et al., 1997) and dog (Mosher et al., 2007). GDF11 also appears to play a crucial role in skeletal development, as knock-out mice display skeletal defects resulting from abnormal anterior–posterior patterning (McPherron et al., 1999).

The expression of *WFIKKN2* in different tissues has been described in humans and mice. In the developing human foetus, *WFIKKN2* is expressed primarily in skeletal muscle (Trexler et al., 2002), and high levels of expression of *WFIKKN2* have also been reported in the skeletal muscle of mice (Hill et al., 2003; Lee and Lee, 2013; Marcelo et al., 2009). Haidet et al. (2008) found that in vivo viral delivery of a *WFIKKN2* expression cassette into adult mice hind-limb and fore-limb muscle significantly increased overall body mass and the mass of the *Tibialis anterior*, *Gastrocnemius*, *Quadriceps* and *Triceps* muscles.

Monestier et al. (2012) observed that the over-expression of *WFIKKN2* caused obvious increases in muscle mass resulting from hypertrophy rather than hyperplasia in a gain-of-function transgenic mouse model, and Lee and Lee (2013) further confirmed the effect of WFIKKN2 on muscle weight, describing how mice genetically engineered to lack *WFIKKN2* had a shift in muscle fibre type from fast glycolytic typeIIb to fast oxidative typeIIa, as well as having impaired muscle regeneration ability. Taken

Abbreviations: PCR, polymerase chain reaction; SSCP, single-stranded conformational polymorphism; GASP1, growth and differentiation factor associated serum protein-1; GDF, growth and differentiation factor; BMP, bone morphogenetic protein; SPR, surface plasmon resonance; SIL, sheep improvement limited.

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together, this literature suggests that WFIKKN2 may be important in the regulation of muscle mass.

Variation in the WFIKKN2 gene has been recorded for humans, cattle, pigs and chickens based on analysis of DNA sequences in GenBank and Ensembl, although the sequence variations observed have not been confirmed in published reports. This suggests that ovine *WFIKKN2* may be polymorphic and thus the objective of this study was to identify if variation occurs in ovine *WFIKKN2*, and in anticipation of a much larger study investigating whether variation in *WFIKKN2* if found, has an effect on ovine growth and muscle traits.

2. Materials and methods

2.1. Sheep investigated and DNA collection

A total of one hundred not closely related sheep, selected from a variety of common breeds in New Zealand (NZ) including Corriedale ($n = 12$), Dorper ($n = 16$), Dorset Down ($n = 16$), Poll Dorset ($n = 18$), Perendale ($n = 16$) and Merino ($n = 22$), were used initially to identify variation in portions of intron 1 and the 3′ UTR of ovine *WFIKKN2*.

Having identified variation in intron 1 of *WFIKKN2*, 487 sheep from eight breeds were used to calculate variant frequency. These comprised 422 sheep from 29 farms in New Zealand, including Corriedale ($n = 48$), Dorper ($n = 60$), Dorset Down ($n = 37$), Poll Dorset ($n = 33$), Perendale ($n = 21$), Merino ($n = 62$) and NZ Romney ($n = 161$), plus 65 Tibetan sheep samples from three farms in the Gansu Province of China. All these sheep were not closely related with the exception of NZ Romneys that were the progeny of five not closely related NZ Romney rams. Genomic DNA was purified from blood collected on FTA cards (Whatman BioScience, Middlesex, UK) according to the method described by Zhou et al. (2006).

2.2. PCR amplification and SSCP analysis

Two sets of PCR primers were designed for amplification of either a portion of intron 1 or the 3′ UTR of ovine *WFIKKN2*, based on the bovine gene (ENSBTAG00000000731). These primers are described in Table 1. The primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA).

Amplifications were performed in a 20 μL reaction consisting of the DNA on one 1.2 mm punch of FTA card, 2.5 μL of 10× PCR buffer (Qiagen, Hilden, German), 0.25 μM of each primer, 150 μM dNTPs (Bioline, London, UK), 2.5 mM Mg^{2+} (Qiagen), 0.5 U Taq DNA polymerase (Qiagen) and ddH_2O to make up volume. The thermal profile for the two regions amplified consisted of 2 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at annealing temperature shown in Table 1 and 30 s at 72 °C, with a final extension of 5 min at 72 °C. Amplification was carried out in Bio-Rad S1000 thermal cyclers (Bio-Rad, Hercules, CA, USA).

The PCR amplicons were visualized by electrophoresis in 1% agarose (Quantum Scientific, Queensland, Australia) gels, using 1× TBE buffer [89 mM Tris (Thermo Fisher, MA, USA), 89 mM boric acid (Thermo Fisher), 2 mM Na_2 EDTA (Thermo Fisher)] containing 200 ng/mL of ethidium bromide (Bio-Rad). A 2 μL aliquot of PCR product was added to 2 μL of loading dye [0.2% bromophenol blue (Bio-Rad), 0.2% xylene–cyanol (Thermo Fisher), 40% (w/v) sucrose (Quantum Scientific)] and the gel was run at a constant 10 V/cm for 10 min, prior to visualization by UV transillumination at 254 nm.

For SSCP analysis, a 0.7 μL aliquot of each amplicon was mixed with 7 μL of loading dye [98% formamide (Fisher Scientific, Loughborough, UK), 10 mM EDTA (Thermo Fisher), 0.025% bromophenol blue (Bio-Rad), 0.025% xylene–cyanol (Thermo Fisher)] and after denaturation at 95 °C for 5 min, samples were rapidly cooled on ice and then loaded on 16 × 18 cm, 14% acrylamide:bisacrylamide (37.5:1) (Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad) for 19 h in 0.5× TBE at conditions described in Table 1. Gels were silver-stained according to the method of Byun et al. (2009).

2.3. Sequencing of amplicons

Amplicons that were identified as homozygous by PCR-SSCP were directly sequenced at the Lincoln University DNA Sequencing Facility. Those sequences only found in a heterozygous form were sequenced using a rapid sequencing approach that has been described previously (Gong et al., 2011).

2.4. Sequence analyses

Hardy–Weinberg equilibrium was calculated using POPGENE version 3.2 (Molecular Biology and Biotechnology Centre, University of Alberta, Canada). Sequence alignment and comparison were carried out using DNAMAN version 5.2.10 (Lynnon BioSoft, Vaudreuil, Canada). The BLAST algorithm was used to search the NCBI GenBank (http://www.ncbi.nlm.nih.goc/) databases for homologous sequences.

A neighbour-joining phylogenetic tree was constructed on the basis of genetic distances in intron 1 of ovine *WFIKKN2*, estimated by the Kimura two-parameter method, using MEGA version 4.0 (Tamura et al., 2007). The reliability of the trees was estimated by bootstrap confidence values, and 1000 bootstrap replications were used.

Sequences for phylogenetic analysis included horse (ENSECAG00000015064), pig (ENSSSCG00000017557), dog (ENSCAFG00000017224) and bovine (ENSBTAG00000000731), together with the ovine *WFIKKN2* variant sequences identified in this study. The sequences were trimmed to a length corresponding to the PCR amplicon before generating the neighbour-joining tree.

3. Results

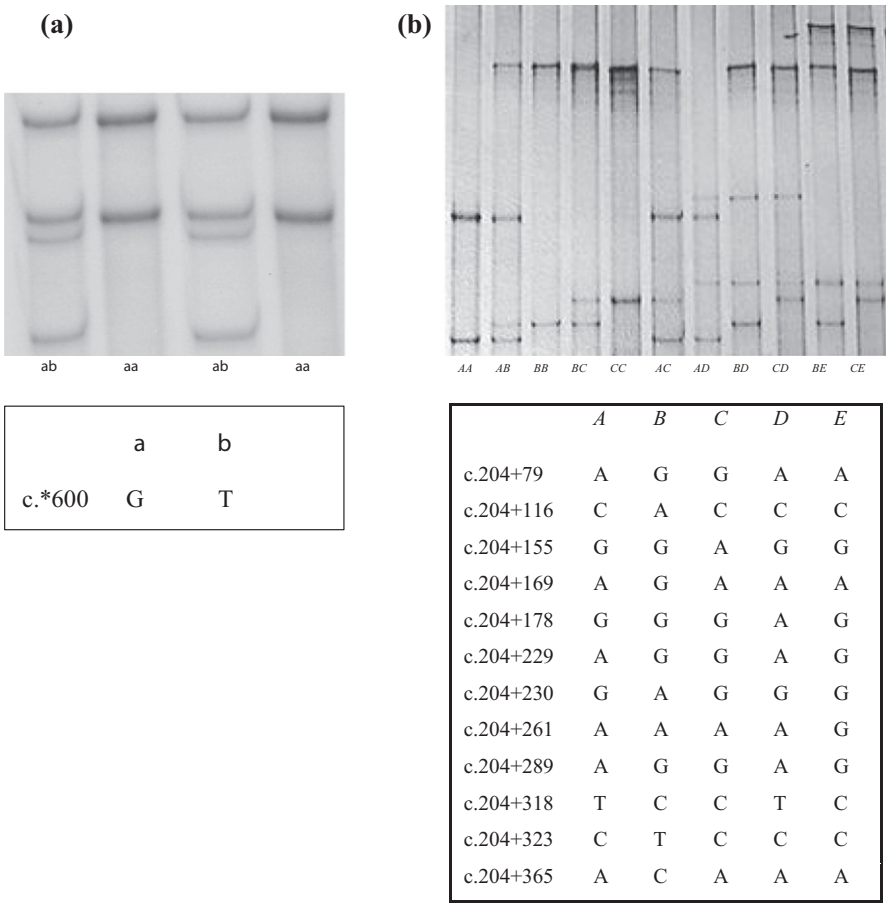
3.1. Identification of variants of ovine WFIKKN2

An amplicon of 399-bp from the 3′ UTR were obtained from all 100 samples from the initial six breeds. Two unique PCR-SSCP patterns (Fig. 1a) representing two unique DNA sequences (designated *a* and *b*) were detected, but only in the Poll Dorset samples. A BLAST search of GenBank revealed that the two variant sequences showed high similarity to the *WFIKKN2* sequences reported from cattle, Tibetan antelope and horse. Variant *a* was identical to the ovine genome assembly v3.1, while *b* had one nucleotide difference to it. These ovine sequences were deposited into GenBank with accession numbers KF824754–KF824755. One single nucleotide polymorphism (SNP) (c.*600G>T) was observed. There were 17 *aa* (94.44%) and 1 *ab* (5.56%) in the original 18 Poll Dorsets, with a frequency of 97.22% and 2.78% for *a* and *b* respectively.

Table 1
The primer sequences and SSCP conditions for amplicons of ovine *WFIKKN2*.

	Primer binding region[a]	Primer sequence (5′–3′)	Amplicon size	PCR annealing temperature	SSCP condition
Intron 1	g.1000_1018	GAGACGGACCAGGTGAGTG	421 bp	58 °C	220 V, 14%, 25 °C
	g.1391_1410	AGAGGCTGGATGAAGCATCG			
3′ UTR	g.5522_5542	AGCACTATGAACAGAGACCAC	399 bp	58 °C	220 V, 14%, 33 °C
	g.5901_5920	TTATCTGCTTCTGGGAGAGC			

[a] The primer binding position is given relative to the bovine *WFIKKN2* DNA sequences (ENSBTAG00000000731).



	A	B	C	D	E
c.204+79	A	G	G	A	A
c.204+116	C	A	C	C	C
c.204+155	G	G	A	G	G
c.204+169	A	G	A	A	A
c.204+178	G	G	G	A	G
c.204+229	A	G	G	A	G
c.204+230	G	A	G	G	G
c.204+261	A	A	A	A	G
c.204+289	A	G	G	A	G
c.204+318	T	C	C	T	C
c.204+323	C	T	C	C	C
c.204+365	A	C	A	A	A

Fig. 1. PCR-SSCP banding patterns and SNPs detected for ovine *WFIKKN2*. (a) Two unique patterns corresponding to two variant sequences (*a* and *b*) in the 3′ UTR. (b) Five unique patterns corresponding to five variant sequences (*A*–*E*) in intron 1. The coordinates of the SNPs are annotated below the patterns based on the bovine *WFIKKN2* sequence (ENSBTAG00000000731) and the numbering of SNPs follows the guidelines at http://www.hgvs.org/mutnomen/.

Overall in the 100 sheep, *aa* (99%) and *ab* (1%) were observed, with frequencies of 99.5% and 0.5% for *a* and *b* respectively.

In a 421-bp amplicon from intron 1 of ovine *WFIKKN2*, five unique PCR-SSCP patterns (Fig. 1b) representing five DNA sequences were identified in the 100 sheep. These sequences were named *A*–*E* and deposited into the GenBank with the accession numbers KF824756–KF824760. None of these sequences was identical to the ovine genome assembly v 3.1, but all shared over 98% homology to it. There were a total of twelve SNPs identified among the five variants (c.204 + 79G>A, c.204 + 116C>A, c.204 + 155G>A, c.204 + 169A>G, c.204 + 178G>A, c.204 + 229G>A, c.204 + 230A>G, c.204 + 261A>G, c.204 + 289G>A, c.204 + 318C>T, c.204 + 323C>T and c.204 + 365A>C, see Fig. 1b). Phylogenetic analysis of these intron 1 sequences and sequences from cattle, pig, horse and dog, revealed that the five variant sequences shared high similarity with the *WFIKKN2* sequences from cattle and other animal species. The variant sequences of ovine *WFIKKN2* could be clustered into two clades, with *B* belonging to one clade and the remaining variants in the other clade (Fig. 2).

3.2. Variant frequencies and Hardy–Weinberg equilibrium for intron 1 in the 487 sheep from eight breeds

The frequencies of the variants of intron 1 of ovine *WFIKKN2* in the 487 sheep from eight breeds are summarised in Table 2. Overall, *B* and *C* were the most common variants in the study, with an overall frequency of 30.60% and 43.33%, respectively, followed by *A* with an overall frequency of 21.25%. *D* and *E* were the least common variants with an overall frequency of 2.46% and 2.46% respectively. The genotype distributions for intron 1 displayed deviation from Hardy–Weinberg equilibrium in the NZ Romney sheep ($P < 0.01$), but were in equilibrium in all of the remaining breeds ($P > 0.05$) (Table 2).

4. Discussion

This is the first study to report sequence variation in ovine *WFIKKN2*. As no complete ovine *WFIKKN2* sequence was yet available when the study commenced, we had to design primers to amplify two regions (intron 1 and the 3′ UTR) of the gene based on the published bovine sequences. In both regions investigated, either one or a combination of two different SSCP patterns was observed for each sheep, which was consistent with there being homozygous and heterozygous genotypes,

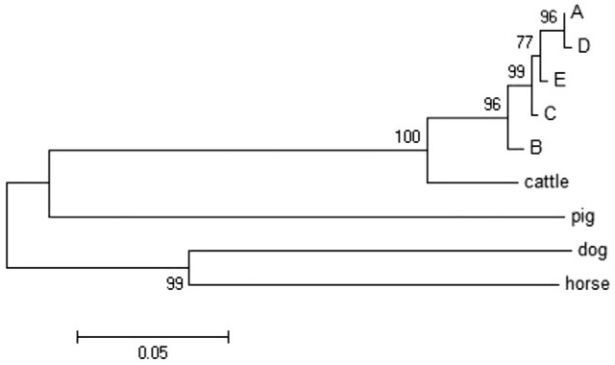


Fig. 2. Neighbour-joining tree for intron 1 sequences of *WFIKKN2* from sheep, cattle, pig, dog and horse. The numbers at the forks indicate the bootstrap confidence values and branch lengths are proportional to genetic distance.

Table 2
Variant frequencies for intron 1 of ovine *WFIKKN2* in various breeds.

Breeds	*n*	Variant frequency (%)					HWE [a]
		A	*B*	*C*	*D*	*E*	
NZ Romney	161	31.37	54.97	11.18	0.93	1.55	0.00
Merino	62	4.84	10.48	70.97	2.42	11.29	0.44
Poll Dorset	33	9.09	9.09	80.30	1.52	0.00	0.94
Corriedale	48	28.12	13.54	51.04	6.25	1.04	0.77
Perendale	21	19.05	35.71	40.48	0.00	4.76	0.61
Dorper	60	35.83	13.33	48.33	1.67	0.83	0.78
Dorset Down	37	20.27	8.11	70.27	1.35	0.00	0.20
Chinese Tibetan sheep	65	0.00	40.00	53.08	6.15	0.77	0.85
Overall	487	21.25	30.60	43.33	2.46	2.46	

[a] *P* value for testing Hardy–Weinberg equilibrium (HWE).

respectively. All the sequences identified shared high homology to the *WFIKKN2* sequences from cattle and other animal species. These results suggest that these sequences represent variants of the ovine WFIKKN2 gene, and that sheep have a single *WFIKKN2* gene, rather than the two *WFIKKN2*-related genes reported in fish genomes (Kondas et al., 2008).

Five and two variants were identified in intron 1 and the 3′ UTR of *WFIKKN2* respectively, suggesting that the gene is polymorphic in sheep. Given that only a portion of intron 1 or the 3′ UTR was amplified, more variants probably exist when an extended gene region is analysed, and further variation should be in linkage with that reported here. It should also be noted that the majority of the sheep investigated were sourced from within New Zealand, with the exception of Chinese Tibetan sheep. It could therefore be expected that different variants may be found when additional sheep from more breeds around the world are investigated.

It is noteworthy that there were twelve SNPs in the 421-bp amplified region of intron 1. When these DNA sequences were aligned, it was revealed that five SNPs (c.204 + 116C>A, c.204 + 169A>G, c.204 + 230A>G, c.204 + 323C>T and c.204 + 365A>C) were in linkage and were unique to variant *B*. In contrast, c.204 + 155G>A, c.204 + 178G>A and c.204 + 261A>G were unique to *C*, *D* and *E*, respectively. There was only a single nucleotide difference between *A* and *D* (c.204 + 178G>A). When compared to the bovine *WFIKKN2* DNA sequence (ENSBTAG00000000731), there was a deletion (c.204 + 110delG) and an insertion (c.204 + 324_c.204 + 325insCTGCATGGAAG) in the ovine variants of intron 1, suggesting that these may be sequence differences between cattle and sheep.

Although the variation identified here is located in intron 1, it may be linked to other variations in critically important regions of the gene that regulate expression. The first intron of other genes has been postulated to modify expression levels and affect transcription efficiency by means of possessing important regulatory elements, such as enhancers, silencers or other elements that modulate the function of the main upstream promoter (Chorev and Carmel, 2012; Gaunitz et al., 2004; Greenwood and Kelsoe, 2003). Furthermore, the association between variation in intron 1 with variation in livestock traits has been reported previously (Hickford et al., 2010; Zhang et al., 2012).

In the two regions studied, polymorphism in the 3′ UTR was low with *a* and *b* having an overall frequency of 99.5% and 0.5% respectively. Given this low variation, only the initial 100 samples were analysed for variation in the 3′ UTR. Based on the variants identified in both intron 1 and the 3′ UTR in the 100 samples typed first, seven haplotypes of ovine *WFIKKN2* could be deduced. These were *Aa*, *Ba*, *Ca*, *Da*, *Ea*, and *Ab* and *Cb*.

It is notable that of the eight sheep breeds investigated for intron 1 variation, only NZ Romney sheep failed to meet the requirement of being in Hardy–Weinberg equilibrium ($P < 0.01$). This may be a consequence of strong artificial selection, as the 161 Romneys studied were progeny of sires, which are ranked in the top 20% of NZ Romney rams in New Zealand based on the Sheep Improvement Limited (SIL — a Division of Meat and Wool NZ, Wellington, NZ)-Dual Purpose Overall (DPO) index. This index is based on measuring genetic merit for a variety of traits of value and has a widespread use in the NZ sheep breeding industry.

The *C* variant was the most common in Merino, Poll Dorset and Corriedale sheep. While the Corriedale sheep is known to have been bred in North Canterbury, New Zealand from Merino sheep, the origin of the Poll Dorset is less certain. It is speculated that Merino sheep and horned Dorset sheep share an ancestral link, based on the observation that both have an extended breeding season. In contrast, while the Perendale sheep is known to be a Cheviot–Romney cross, the variant frequencies in the two breeds appear to be quite different, with *A* and *B* as the most common variants in the NZ Romney sheep, while in Perendale sheep the most common variants were *B* and *C*. Care is needed in this interpretation as the sheep typed were not chosen so as to necessarily be representative of the breeds as a whole.

Some variants were not found in some of the breeds. In the 3′ UTR region, the *b* variant was only found in Poll Dorset sheep. Care is needed in pronouncing this variant to be breed-specific as once again the sheep investigated were not necessarily representative of all worldwide sheep breeds. In intron 1, compared with a relatively high frequency of *A* in the NZ breeds, there was an absence of *A* in the Chinese Tibetan sheep. This too may be breed-specific, as may be the lack of *D* and *E* in Perendales, Poll Dorsets and Dorset Down sheep, although only small numbers of animals from each breed were studied.

Although no obvious patterns in variant frequency were observed with respect to the recognised wool breed (Merino), meat breed (Poll Dorset, Dorper and Dorset Down) or dual-purpose breed (Romney, Corriedale, Perendale and Chinese Tibetan sheep), the distribution of individual variants seems to be related to generalised production traits in some breeds. For example, *E* was the second most common variant (11.29%) in Merino sheep, while it was rare in the other breeds (0.00–4.76%). It may therefore be associated with unique traits (such as wool traits) in Merino sheep. WFIKKN2 can bind bone morphogenetic protein 2 (BMP2) and bone morphogenetic protein 4 (BMP4) with relatively high affinity (Szlama et al., 2010), and the genes for these two proteins are expressed in hair shaft precursor cells (Kulessa et al., 2000), while ectopic expression of *BMP2* or *BMP4* suppresses the formation of feather buds in chick embryos (Jung et al., 1998; Noramly and Morgan, 1998). Additionally, because WFIKKN2 possesses a follistatin-domain, it may have an overlapping function with follistatin, which has been shown to be an important regulator of cell proliferation, differentiation and apoptosis in hair follicle initiation, hair cycling, normal skin homeostasis and wound healing (McDowall et al., 2008). This speculation would need to be investigated by looking for associations between *WFIKKN2* variation and variation in wool traits.

Given that WFIKKN2 interacts with GDF8 and GDF11, and because these factors play significant roles in the regulation of muscle development, it might be inferred that WFIKKN2 has an effect on growth or muscle traits in sheep. In this respect it is notable that *A* exists at a higher frequency (4.84–35.83%) in the NZ sheep, but is absent in the Chinese Tibetan sheep. Chinese Tibetan sheep tend to be slower growing than NZ sheep because of feed availability in the China–Tibetan plateau region, but this may also reflect genetic differences including differences in *WFIKKN2*. This too will require much additional investigation with more sheep and breeds. The *A* variant was also not present at high frequency in Merino sheep, a breed that is slow growing in the NZ context (Muir et al., 1992). Together, this suggests that *WFIKKN2* is worthy of further study in the context of variation in growth and muscle traits.

Conflict of interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2014.03.062.

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