Expression of the developmental regulators *Msx1* and *Msx2* in sheep skin varies with body region and wool growth pattern

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ABSTRACT

The variability of fibre characteristics affects the processing performance of wool and therefore the value of the wool clip. These variations originate during development *in utero*, and manifest as patterns of fibre growth that differ between body regions. Two transcription factors, Msx1 and Msx2, have previously been linked to regional specification within the skin and also act as regulators of differentiation within the hair follicle matrix. In this study, we describe the expression patterns of *Msx1* and *Msx2* in multiple body sites of foetuses and adult crossbreed ewes. In foetal skin, the expression of both *Msx1* and *Msx2* was greatest in the regions of lower eventual follicle density. In adults, both anterio-posterior and dorso-ventral gradients were observed in *Msx1* expression across the fleece bearing skin. Higher concentrations of *Msx1* and *Msx2* transcripts were present in adults than foetuses, possibly reflecting additional roles in regulating the differentiation and growth of the wool fibre shaft. These results are consistent with the involvement of Msx1 and Msx2 in the development of skin regions, wool follicle growth, and anterio-posterior and dorso-ventral gradients of wool characteristics in sheep.

Keywords: Msx1; Msx2; wool; hair; sheep; topobiology; morphogenesis.

INTRODUCTION

Variations within and between wool fibres of a harvested fleece undermine the value of the wool. Variable attributes include and fibre diameter, staple length and the pattern of crimping (Craven *et al.*, 2007; Sumner & Craven, 2000; Young & Chapman, 1958). This variation arises both within and between fibres in the same staple. Variations between differing body regions have been shown to contribute up to one third of the variation within a Romney fleece (Sumner & Revfeim, 1973). Furthermore, the costs associated with low value fibres outside the prime fleece regions substantially reduce the wool growers' current slim profit margins. Long belly wool is of low commercial value and significantly increases the time taken to shear an animal (Scobie *et al.*, 2006). Unwanted long fleece cover around the breech necessitates crutching or dagging to reduce the incidence of dagginess and flystrike in the live animal and microbial contamination of sheep carcasses during meat processing (Fisher *et al.*, 2004; Scobie *et al.*, 2006).

Wool attributes are determined by the growth of follicles which develop *in utero* as a consequence of interactions between the epidermis and the underlying dermis (Millar, 2005). These processes commence at approximately 60 days of gestation in sheep (Moore *et al.*, 1998). Importantly, it is the properties of the dermis that are predominant in determining the type of follicle formed (Sengel, 1990). In vertebrate embryos, the dermis of dorsal, ventral and head regions derive from distinct origins (Dhouailly *et al.*, 1998; Sengel, 1990). Head dermis mostly differentiates from neural crest cells. Ventral and lateral dermis of the trunk originates from the somatopleure, whereas the dorso-lateral dermis derives from the dermamyotomal cells of the somites. Thus, the gross differences in pelage between face, back and belly correlate with the different embryonic origins of the dermis.

Homeobox genes are major regulators of spatial patterning during development. They can be divided into two large families: a set of 39 canonical *Hox* genes involved in patterning the whole embryo, and a large more diverse family of non-*Hox* homeobox genes which are dispersed throughout the genome and appear to be generally involved in development and patterning within specific organs (Cillo *et al.*, 2001). Examples of non-*Hox* homeobox genes are *Msx1* and *Msx2* which are related to the *Drosophila* gene *Msh* (muscle segment homeobox) (Davidson, 1995). Msx genes generally act as repressors of differentiation and modulators of cell adhesion (Cillo *et al.*, 2001). Their patterns of expression suggest that they are involved in patterning of the anterior-posterior (A-P) axis of the body, organogenesis, the elongation of limbs and organs and a range of other developmental processes (Suzuki *et al.*, 1997) including ventralisation of the body wall (Ogi *et al.*, 2005; Suzuki *et al.*, 1997).

Msx1 and *Msx2* have also been shown to be involved in both dermal specification (Houzelstein *et al.*, 2000) and hair follicle growth (Ma *et al.*, 2003; Satokata *et al.*, 2000). Msx1 is involved with the induction of the dermis in a region-specific manner, and is thought to act as a repressor of differentiation (Houzelstein *et al.*, 2000). *Msx1* and *Msx2* are known downstream targets of bone morphogenetic proteins (BMP) which are key agents in the formation, spacing and polarity of developing follicles, especially secondaries (Botchkarev *et al.*, 2002). In the mouse, *Msx1* and *Msx2* are expressed in the hair follicle placode and subsequently in the epithelial matrix cells. Both genes are later expressed in the matrix cells of the hair bulb during anagen where Msx2 is involved in the differentiation of the hair shaft. Mice deficient of both *Msx1* and *Msx2* have only one third of the normal number of hair follicles (Satokata *et al.*, 2000), demonstrating the requirement for Msx protein in normal follicle induction. Although Msx2 may add to, and compensate for, the influence that Msx1 exerts during follicle morphogenesis, its primary function in the mature follicle is to regulate transitions between follicle growth phases by maintaining anagen and to regulate hair shaft differentiation (Ma *et al.*, 2003). Thus, these genes are candidates for controlling aspects of dermal development, epithelial differentiation, and continued epithelial proliferation into adulthood (Stelnicki *et al.*, 1997) and may therefore influence structural features of the sheep fleece.

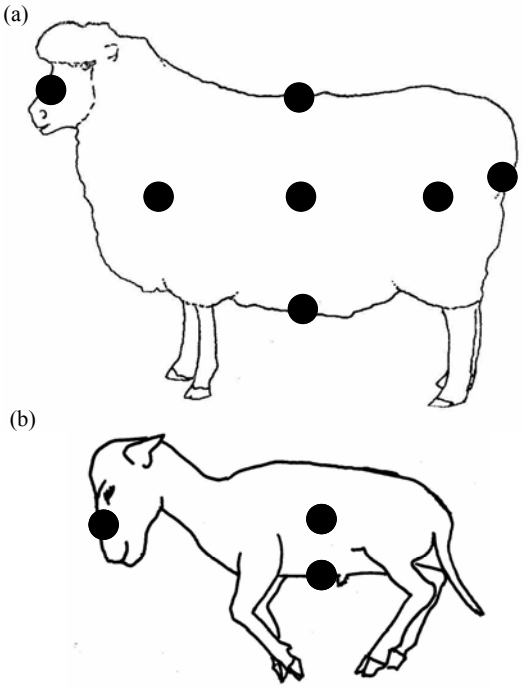
If Msx genes are involved in patterning follicle development and wool growth, one might expect differences in expression during early follicle formation in the foetus and perhaps also in adults. In this study, we set out to investigate whether the mRNA expression of *Msx1* and *Msx2* in sheep skin differs between body regions. We describe the mRNA expression profiles of *Msx1* and *Msx2* in foetal sheep skin at a time when wool follicle morphogenesis is occurring. We show that mRNA levels vary with the body sites and developmental stages. Both *Msx1* and *Msx2* expression is maintained into adulthood. Variations between body site and sheep strains are associated with differences in fleece characteristics.

METHODS

Animals

Skin samples were collected from sheep with either large or small dorso-ventral differences in fibre length such that they could be characterised as either "bare" (B) or "woolly" (W) bellied phenotypes. These sheep were developed in a selective breeding programme (Scobie *et al.*, 1997; Scobie *et al.*, 2006). The oestrus cycles of ewes were synchronised using controlled internal drug releaser (CIDR) devices, and mating conducted as part of normal flock management. Four ewes of each phenotype (B and W) were sacrificed at day 70 of gestation and two of each phenotype at day 90 of gestation. Skin was collected from the midside, belly and face of foetuses, and from seven body sites of the ewes (Figure 1). Sub-samples were preserved in either 10% phosphate buffered formalin or liquid nitrogen for later analysis of skin structure and gene expression. In addition, tissue compartments were isolated from whisker follicles of abattoir slaughtered sheep for RNA analysis. Follicles were micro-dissected to separate outer root sheath, germinal epithelium and dermal papilla as described elsewhere (Rufaut *et al.*, 2007; Rufaut *et al.*, 2006).

Figure 1: Sampling sites of (a) adult and (b) foetal sheep.



RNA analysis and real time polymerase chain reaction (PCR)

Whole skin tissue was ground in a freezer mill, and total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). This RNA was further purified, DNase treated, and reverse transcribed using Superscript III (Invitrogen) with random hexamers and 1.0 μg of RNA. PCR primers were designed using Primer3 software. Sequences were as follows:

Msx1 forward 5'-CATTTCTCGGTGGGAGGAC-3',
Msx1 reverse 5'-GGGTCCTCTCGGGTTTCTC-3',
Msx2 forward 5'-CTGGTCAAACCCTTCGAGAC-3',
Msx2 reverse 5'-AGTATCTGCCCGGTTCCTG-3'
yielding PCR products of 44 and 46 base pairs respectively. Individual results were normalised to expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward primer 5'-CGCCAAGAAGGTCATCATCTCTGC-3' and reverse primer 5'-GCGTGGACAGTGGTCATAAGTCCC-3', yielding a 195 bp amplicon). Measurement of gene expression was performed by real time PCR on a Lightcycler II (Roche, Mannheim, Germany) using Sybr green Mastermix reagents (Roche) and 2 μL of 1 in 10 dilution of cDNA. PCR amplification was carried out over 40 cycles of 95°C denaturation, 60°C annealing and 72°C extension and concentrations to achieve threshold levels compared to a standard curve.

Statistical analysis

Relative expression levels of Msx1 and Msx2 (normalized to the internal control gene GAPDH) were log transformed and within-sheep site variations and between-selection line differences determined using a general linear model analysis (GenStat Committee, 2005).

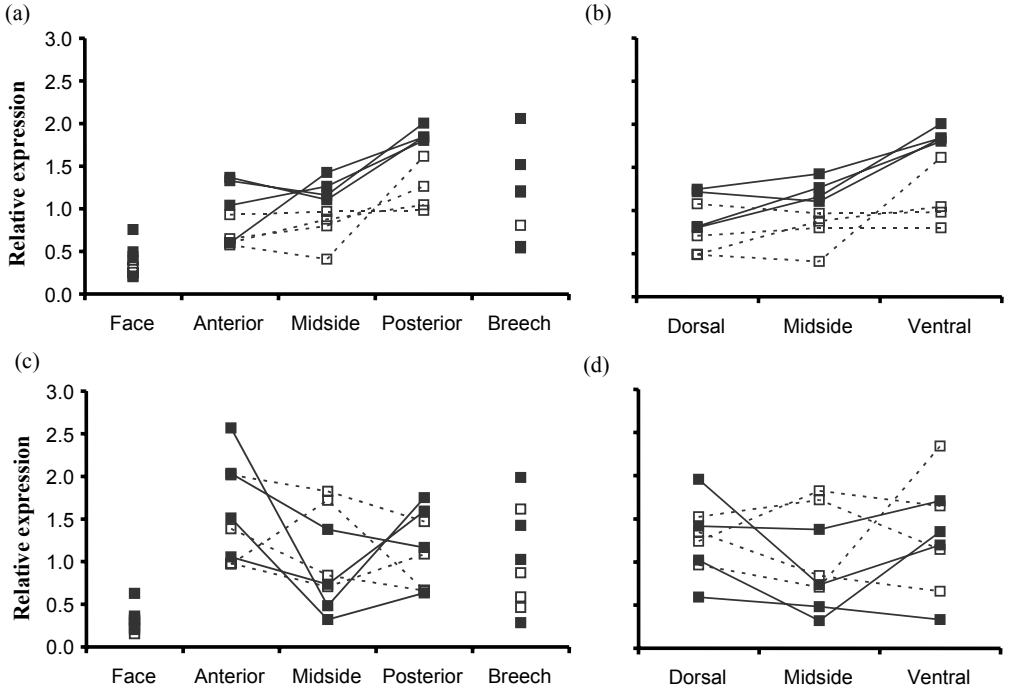
RESULTS

Msx1 and *Msx2* mRNAs were detected readily in both adult and foetal skin by real time PCR. In adult sheep skin, both anterio-posterior and dorso-ventral gradients were observed in *Msx1* expression (Figure 2). Posterior and ventral sites had higher levels of *Msx1* mRNA than anterior ($P<0.01$) and dorsal ($P<0.001$) sites respectively. Furthermore, *Msx1*, mRNA was 4-fold lower on the face than the belly ($P<0.001$). Overall, the bare bellied group (B) of sheep contained 1.5 fold ($P<0.01$) more *Msx1* mRNA than those with woolly bellies (W). Within-animal gradients were similar in the two groups.

Concentration of *Msx2* was approximately 10-fold greater than *Msx1* as indicated by fewer cycles of PCR required to achieve threshold levels. However, except for a 4-fold lower concentration in facial skin ($P<0.001$), no site or strain differences in *Msx2* mRNA levels were observed, with large variation in levels between samples being characteristic (Figure 2).

In whole foetal skin at 70 days gestation, the expression of both *Msx1* and *Msx2* were higher on the belly than the midside (*Msx1*, $P<0.01$; *Msx2*, $P<0.05$). On the face, in contrast to adult skin, expression of *Msx1* was five-fold ($P<0.001$) higher

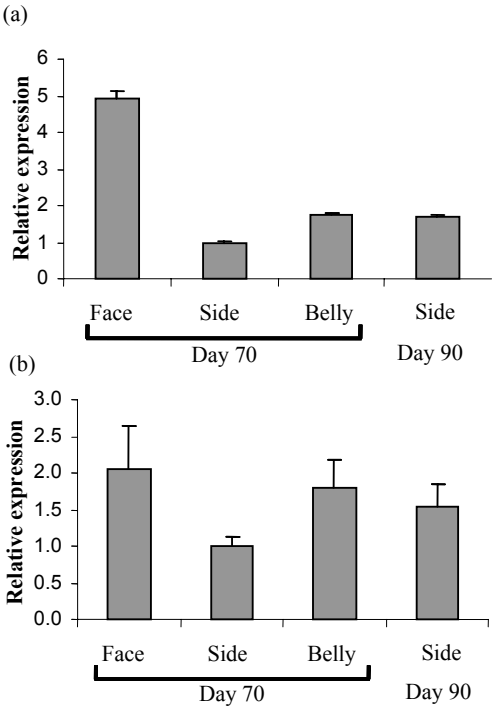
Figure 2: Relative levels of Msx1 ((a) and (b)) and Msx2 ((c) and (d)) mRNA in adult skin across the body. Anterio-posterior ((a) and (c)) and dorso-ventral ((b) and (d)) comparisons between woolly-bellied (□) and bare-bellied (■) sheep are shown. Individual mRNA levels are normalised to GAPDH and shown relative to mean midside concentrations.



than the midside and *Msx 2* was two-fold ($P<0.05$) higher (Figure 3). By 90 days gestation, levels of both *Msx1* and *Msx2* in mid-side skin had increased ($P<0.01$) with further wool follicle development in the skin. No differences in skin *Msx1* or *Msx2* mRNA levels were found between foetuses from woolly and bare bellied ewes.

Similar ratios between *Msx1* and *Msx2* transcripts were observed in adult and foetal skin, however higher concentrations of up to 10-fold, of both were present in adults, possibly reflecting additional roles in regulating the differentiation and growth of the wool shaft.

Figure 3: Relative levels of (a) Msx1 and (b) Msx2 mRNA in foetal skin varies with site and developmental stage. Site and developmental stage (Day 70 and day 90 of gestation) comparisons of all sheep are indicated. Individual levels are normalised to GAPDH and shown relative to mean midside concentrations. Error bars indicate the standard error of the mean (SEM).



While our data on the regional variation in Msx expression are derived from extracts of whole skin, we have also begun to investigate the localisation *Msx1* and *Msx2* transcripts by comparing their relative abundance in micro-dissected compartments of ovine whisker follicles. Both *Msx1* and *Msx2* transcripts were most plentiful in the lower bulb, including the dermal papilla and the germinal epithelium, which contained greater concentrations of Msx mRNA than outer root sheath keratinocytes explants (Craven, A.J. unpublished data).

DISCUSSION

We have shown that the developmental regulators *Msx1* and *Msx2* are expressed in sheep skin at critical times of wool follicle development and growth. Furthermore gradients of Msx1 mRNA both across and down the fleece-bearing regions were evident. As such, there is a negative correlation of patterns of *Msx1* expression with wool characteristics across fleece, as reported in breeds ranging from Merinos (Young & Chapman, 1958), Romneys (Sumner & Revfeim, 1973), Perendales (Sumner & Craven, 2000) and Wiltshires (Craven *et al.*, 2007). Furthermore, bare-bellied sheep consistently had higher levels of *Msx1* expression across all body sites. Thus it is possible Msx1 may help determine the short-woolled belly phenotype exhibited by these sheep.

The face of adults had lower levels of both *Msx1* and *Msx2* mRNA as compared to the fleece regions. With Msx expression being localised to follicles, this may reflect the lower follicle density found on the face. In addition, facial follicles undergo significant periods of quiescence, and so differences between the face and fleece may also reflect differences in follicle growth cycling.

As the characteristics of the wool follicle are established during development, we looked to see if levels of Msx mRNA varied at times follicle initiation. At 70 days of gestation, when primary follicles are developing, higher levels of both *Msx1* and *Msx2* expression occurred in regions of lower follicle density and eventual wool growth. On the other hand, differences between sites in developmental timing may complicate interpretation of the data. Follicle development begins on the head and spreads over the rest of the body in a progressive wave, moving in an anterior-posterior and dorso-ventral direction. Consequently, the more anterior foetal skin samples may represent more advanced developmental stages, and so differences in Msx expression could reflect differences in follicle density as well as mRNA abundance within follicles. In particular, this may explain the contrast between high facial expression in the foetuses and low facial expression in the adults. On the midside at day ninety of gestation, levels of both Msx1 and Msx2 mRNA had increased. Again, this may reflect increased follicle density attributable to the secondary follicles which are beginning to form. To resolve these considerations, it would be necessary to evaluate more extensively both the intensity and localisation of Msx

expression throughout development.

Having shown that the expression of Msx1 in particular is associated with patterns of follicle growth, we suggest this gene may be involved in determining variations in fleece cover across the sheep. The question remains, however, whether variations in Msx1 expression are a cause or an effect of the wool growth phenotype. In other species, Msx1 is involved in the formation of both the dermis (Houzelstein *et al.*, 2000) and epidermis (Suzuki *et al.*, 1997) in a regionally-dependent manner. Later in skin development, Msx1 is also expressed during initiation of follicles, when epithelio-mesenchymal interactions induce cells to form placodes (Stelnicki *et al.*, 1997). Our preliminary PCR data suggest that, in adult sheep, Msx1 is expressed in the lower follicle, including the dermal papilla. Bearing in mind the role of the dermis in specifying follicle type (Sengel, 1990), the available expression data are consistent with the idea that Msx1 may play a causative role in the specification of regional variations in the fleece. The phenotype of knockout mice deficient in both *Msx1* and *Msx2*, which have only one third of the normal number of hair follicles (Satokata *et al.*, 2000), further supports the possibility of a causative role.

Confirmation of this role would require manipulation of Msx gene expression in an ovine system, to alter the wool growth phenotype. Although technically demanding, a combination of lentiviral vectors and follicle induction by implanted dermal papilla cells may make such experiments possible in the near future. Alternatively, if quantitative trait loci (QTL) influencing fleece distribution are discovered, it would be interesting to determine whether the loci covered the Msx genes or genes involved in associated pathways. As identified on the Virtual Sheep Genome Browser (version 1.2.1; www.livestockgenomics.csiro.au) *Msx1* is located on the ovine chromosome 6 (OAR6:107611153 to 107615383) while *Msx2* is located on chromosome 16 (OAR16:6244181 to 6250508).

In summary, understanding the expression patterns and cellular functions of Msx genes may help clarify mechanisms underlying the variation in wool growth across the body of the sheep. This information could aid in breeding programmes aimed at producing sheep with a desirable fleece cover.

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