Improved Wool Production in Transgenic Sheep Expressing Insulin-like Growth Factor 1

Sami Damak[1], Hung-yi Su[1,2], Nigel P. Jay[2] and David W. Bullock[1,2,*]

[1]Centre for Molecular Biology and [2]Animal and Veterinary Sciences Group, Lincoln University, PO Box 84, Canterbury, New Zealand.
*Corresponding author (e-mail: bullock@lincoln.ac.nz).

Transgenic sheep were produced by pronuclear microinjection with a mouse ultra-high-sulfur keratin promoter linked to an ovine insulin-like growth factor 1 (IGF1) cDNA. Five transgenic lambs resulted from the microinjection of 591 embryos; one male and one female showed IGF1 expression in the skin. A progeny test of the ram was carried out by matings to 43 non-transgenic ewes. Of 85 lambs born, 43 (50.6%) were transgenic. At yearling shearing (approximately 14 months of age), clean fleece weight was on average 6.2% greater in transgenic animals than in their non-transgenic half-sibs, with a greater effect in males (9.2%) than females (3.4%). Transgenics showed a small but significant increase in bulk, but male transgenics had a lower staple strength than female transgenics and non-transgenics which did not differ significantly. There were no significant differences in fiber diameter, medullation, and hogget body weight. To our knowledge this is the first reported improvement in a production trait by genetic engineering of a farm animal without adverse effects on health or reproduction.

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Despite success in producing transgenic farm animals, improvement of production traits by genetic manipulation has remained elusive. Stemming from our demonstration in the accompanying paper of a mouse keratin promoter driving heterologous gene expression in the wool follicle of transgenic sheep[1], we set out to introduce a gene that could improve wool production or properties. We chose to focus on hormone and growth factor effects, as likely to influence follicular activity.

The literature relating to hormonal effects on wool production in sheep is inconclusive. A positive influence of growth hormone on wool growth was first reported by Wagner and Veenhuizen[2]. Similar effects were later found by several groups[3–4], in some cases during growth hormone treatment[6–8], in others in the post-treatment period[9–11]. Several studies reported no effect[12] or a decrease in wool growth[9–11].

Insulin-like growth factor 1 (IGF1) mediates many of the actions of growth hormone[13,14]. In growth hormone-deficient rats, the primary response to the administration of growth hormone is stimulation of circulatory IGF1[15] and a rapid increase in IGF1 mRNA transcription in the liver[16]. Newborn mice homozygous for targeted disruption of the IGF1 gene show, in some cases lethal, growth retardation[17]. In a convincing demonstration of the ability of IGF1 to substitute for growth hormone, Behringer et al[18] showed that an IGF1 transgene in mice with ablation of pituitary somatotrophs was able to restore growth rate and body weight to normal. IGF1 may have a dual function, acting both as a mitogen and as a morphogen[19,20].

Wool growth in sheep is determined by proliferation and differentiation of epithelial cells in the follicle bulb. A reaction-diffusion system has been proposed to account for the proliferation and commitment of epithelial cells to the cortical region of the developing fiber[22,23].

In the absence of direct or conclusive evidence of an effect of growth hormone on wool growth, the properties of IGF1 as a somatomedin and its dual role in mitogenesis and differentiation led us to consider this growth factor as a potential regulator of wool follicle activity or fiber development. Therefore, we investigated whether overexpression of IGF1 in the wool follicle of sheep might influence wool production or fiber characteristics.

We have shown[1] that a mouse ultra-high-sulfur keratin (KER) gene promoter is capable of directing overexpression of a heterologous gene to the wool follicle in transgenic sheep, in particular to the keratogenous zone of the cortex in the developing fiber. We report here the construction of a KER-IGF1 plasmid and its use to generate transgenic sheep. Progeny tests show an improvement in wool production in sheep expressing the IGF1 transgene.

Results

Transgenic sheep. A DNA construct containing the sheep IGF1 cDNA ligated downstream from a mouse keratin promoter (Fig. 1) was used to produce transgenic sheep. Microinjection and transfer of 591 embryos in 1991 resulted in 103 lambs born, of which 5 were transgenic as determined by Southern blot analysis (not shown), with an estimated 1 to 10 unrearranged copies of the transgene. One ram (Line nine) was mated to 43 random-bred non-transgenic ewes, producing 85 lambs of which 43 (50.6%) were transgenic.

Gene expression. Northern analysis of total skin RNA detected IGF1 expression both in transgenic and non-transgenic animals (not shown). Ribonuclease protection assay (RPA) was thus used to distinguish between endogenous and transgenic expression and to test for correct initiation of transcription. Of the five G0 animals, one male and one female showed a band corresponding to expression of transgenic IGF1 mRNA in skin. Among the G1 transgenic progeny of the expressing ram (Line nine) 20 out of 25 lambs tested showed expression of the IGF1 transgene in the skin (Fig. 2). All animals, including non-transgenic controls, showed endogenous expression of IGF1 as demonstrated by a 200-bp band in the RPA. The level of transgene expression, estimated by the intensity of the band, was about twice that of endogenous expression (Fig. 2).

Progeny testing. To assess phenotypic effects of IGF1 expression in the wool follicle, a progeny test was carried out on the G1 lambs of Line nine, comparing wool production and properties of transgenics with their non-transgenic half-sibs during the lamb year. Twenty-six transgenic lambs survived to yearling shearing; losses were no greater in transgenics than in non-transgenics (26/42 survival, chi-square = 0.019). IGF1

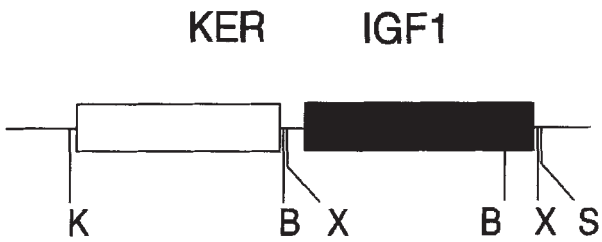

FIGURE 1. The KER-IGF1 construct used to produce transgenic sheep. K: KpnI; B: BamHI; X: XbaI; S: SacI.

expression could not be detected by RPA in four transgenic animals. When fleece-weight data were analyzed, values for these transgenic non-expressors were not significantly different ($p > 0.05$) from non-transgenic half-sibs. Thus, the non-expressors and non-transgenics were pooled in the results presented here (Table 1).

Wool production. Clean fleece weight was determined at yearling shearing (approximately 14 months of age); note that these animals were *not* shorn as lambs. Using means adjusted for body weight as a covariate, wool production on average was 6.2% greater ($p = 0.028$) in transgenics than in their non-transgenic half-sibs. The effect was greater in males (9.2%) than females (3.4%), but the difference between sexes was not statistically significant ($p = 0.252$). There was no significant difference in yield after scouring between transgenics and non-transgenics ($p = 0.919$), but females had significantly cleaner fleeces than males ($p < 0.001$) based on this parameter. Rate of wool growth (g/day) was significantly greater ($p = 0.029$) in transgenic than non-transgenic animals. Transgenic status had no significant effect on body weight ($p = 0.175$), but males were heavier than females ($p = 0.051$).

Wool properties. Mean fiber diameter varied between 36 and 38 microns, which is in the normal range for this coarse-wool breed, and was not significantly different between transgenics and non-transgenics ($p = 0.120$) or between the sexes ($p = 0.418$).

Medullation, a measure of the extent to which the fiber is filled with cells, was also unaffected by transgenic status ($p = 0.779$). However, transgenics showed a small (approximately 1%) increase in bulk, a volume:weight measure related to the "crimp" of the wool, which was statistically significant ($p = 0.042$).

Table 1. Wool production and fiber characteristics at yearling shearing (mean ± sd).

	Transgenic		Non-Transgenic	
	Male	Female	Male	Female
Number of animals	12	10	16	14
Clean fleece weight (Kg)	5.10 ± 0.14	5.14 ±0.17	4.67 ±0.12	4.97 ±0.13
Percent yield	73.24 ±0.98	79.39 ±1.17	73.25 ±0.86	79.53 ±0.90
Rate of wool growth (g/day)	114.3 ±0.30	115.4 ±0.36	104.5 ±0.27	111.4 ±0.28
Fiber diameter (microns)	37.07 ±0.63	38.28 ±0.76	36.73 ±0.56	36.59 ±0.59
Medullation (units)	1.99 ±0.20	1.93 ±0.26	2.10 ±0.17	1.93 ±0.18
Bulk (cm^3/g)	19.83 ±0.25	19.82 ±0.30	19.41 ±0.22	19.15 ±0.23
Staple Strength (Newtons/Ktex)	29.20 ±3.05	47.93 ±3.64	38.96 ±2.69	41.67 ±2.82
Body weight (Kg)	74.00 ±1.47	70.39 ±1.69	75.31 ±1.27	73.09 ±1.36

Average staple strength, determined as the peak force required to break a wool staple expressed per unit of cross-sectional area, was not affected by transgenic status ($p = 0.572$), but there was a significant sex effect ($p = 0.001$) and a marked interaction between sex and status ($p = 0.011$). Transgenic male staple strength was 39% lower than transgenic female, and 28% lower than the average of non-transgenic animals.

Discussion

We have previously shown that this keratin promoter directs gene expression to the keratogenous zone of the wool follicle in transgenic sheep[1]. In the present study, expression of sheep IGF1 driven by the KER promoter increased wool production in transgenic animals compared to their non-transgenic half-sibs. To our knowledge this is the first reported improvement of a production trait by genetic engineering of a farm animal without adverse effects on the animal's health or reproduction.

Wool production, expressed as clean fleece weight, is a function of fiber length, diameter, and number. Fiber number is difficult to determine accurately and studies of follicle density in these animals have not yet been performed. Since there was no significant effect of transgenesis on fiber diameter it is possible that an increase in fiber length, representing an increase in the rate of fiber growth, was responsible for the increase in fleece weight. The average daily wool growth was significantly higher in transgenic animals. Preliminary data indicate that the differential effect of IGF1 was due to a faster rate of wool growth in transgenic sheep during the spring rebound after the seasonal winter decline, which may have been less marked in the transgenic animals (not shown). Our earlier work showed that the activity of the KER promoter correlated with the rate of wool growth[1], and a similar effect was seen in the hair cycle of mice[24].

The rate of fiber growth is determined by the proliferation of cells in the follicle bulb and their subsequent migration and growth[25]. Local infusion of IGF1 into the skin of sheep has been shown not to affect the numbers of replicating cells in the follicle[26]. It is unlikely that epithelial cell proliferation was involved in the increased production due to IGF1 expression, since the KER promoter is active in keratinizing cortical cells after differentiation[1,24]. Harris et al[26] reported that local infusion of IGF1 increased blood flow, oxygen utilization, and amino acid uptake (including cysteine) in sheep skin. While this work was in progress, Philpott et al[27] demonstrated that IGF1 stimulated hair growth in cultured human follicles, but possible mechanisms were not discussed. Attempts have been made to increase wool production by introducing bacterial genes coding for enzymes involved in cysteine synthesis on the basis that availability of cysteine may be limiting fiber growth[28,29]. The fact that IGF1 increased fleece weight suggests that dietary cysteine was not rate-limiting in this study. Although they were physiologically impaired, transgenic sheep expressing growth hormone[30] did show an increase in wool growth (K.A. Ward, personal communication).

The effect is thus likely to be due to a stimulation of follicular metabolism by this growth factor, although indirect effects cannot be excluded. While circulating IGF1 was not determined, it should be noted that body weight was not significantly affected. As somatic growth is a primary response to systemic IGF1[18], body weight would have been expected to increase if activity of the promoter in tissues other than the skin[1] had led to a rise in IGF1 concentration in the blood. Taking this argument together with the direct effect of IGF1 on hair growth *in vitro*[27], a systemic or indirect effect

seems unlikely.

Apart from staple strength, and a small increase in bulk, other fiber characteristics were unaffected by expression of the transgene. Bulk is an important factor in the commercial uses of processed wool and maintenance or slight improvement in this attribute is encouraging. The range of values from 18–21 cm^3/g is normal for this breed, but the average of about 19 would be considered low by commercial standards. No effort was made to select for high quality wool among the embryo donors.

We have no immediate explanation for the lower staple strength among transgenic males. Staple strength is a highly variable measure, in part because it is difficult to ensure that all fibers in the staple are under even tension when mounted in the apparatus used to determine breaking strength. The values obtained ranged from 6 to greater than 50 Newtons per kilotex, but there was no heterogeneity of variance between groups. A reduction in staple strength could be anticipated if an environmental set-back, climatic or nutritional, had occurred to interrupt wool growth and cause a weakness leading to a break-point in the staple. Any such influence, however, would be expected to affect all groups equally, as the animals were run in close proximity and transgenic males were run together with non-transgenic males.

Few investigators have taken account of sex either in IGF1 effects or wool growth. Female hair was used in the *in vitro* studies of IGF on human follicles[27], but the sex of the six sheep used in the local perfusion studies of IGF1 was not recorded[26]. Lack of attention to sex in investigations of wool growth may reflect the assumption that most wool production comes mainly from females (about 80% of New Zealand's national flock of 56 million sheep are ewes). Whether the recorded reduction in staple strength in transgenic males is real will transpire from wool measurements on these animals as adults, and whether the effect is heritable will become evident from their progeny. The circumstances in this work, where both intact males and dry females were examined, are unusual. It should be noted that staple strength was not reduced in transgenic ewes, although the increase in wool production was less in ewe than in ram lambs. The design of this experiment may not reflect the results that might be obtained when this transgene is introduced into a commercial flock, where ewes would normally be mated.

While difficult to quantitate, the fact that transgene mRNA expression was of the same order of magnitude as endogenous IGF-1 skin expression suggests that the activity of the transgene was relatively low. Increases in performance might be achieved by higher levels of expression, for example through breeding to homozygosity or use of a stronger promoter. Improvements might also be achieved by adopting a different approach, such as expressing IGF1 receptor or cysteine t-RNA genes, or using a promoter that expresses in follicle cells at earlier stages of differentiation.

The results demonstrate that heritable, stable expression of IGF1 in the wool follicle of transgenic sheep is capable of increasing fleece weight at yearling shearing. Progeny testing of these two-year-old animals is continuing and results will be reported at a later date.

Experimental Protocol

Animals. Unselected Coopworth (Romney x Border Leicester) ewes of mixed age were used as embryo donors and recipients. Superovulation and embryo transfer were carried out using a protocol for year-round production of pronuclear embryos as described[1]. The animals were run on a rye grass/white clover pasture without supplementary feeding. Applicable regulations relating to DNA manipulation, animal ethics, and field trials of transgenic sheep, under approval from the Ministry for the Environment, were observed. From April to December 1991, 1225 embryos were collected from 185 superovulated ewes and 716 (58%) were injected, of which 591 (83%) were transferred to 116 recipients. Pregnancy was allowed to go to term without intervention, and pregnant ewes were brought indoors about seven days before delivery. At birth, each lamb was identified and an ear biopsy taken for DNA analysis.

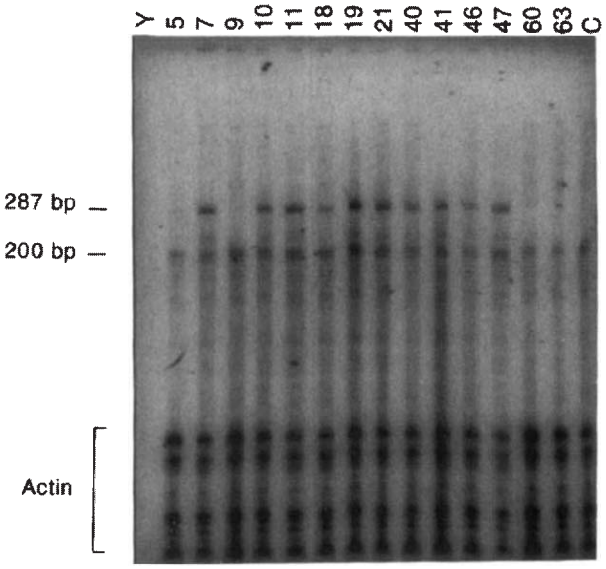

FIGURE 2. Analysis of IGF1 expression in transgenic sheep skin by ribonuclease protection assay. The IGF1 probe protected a 287-bp fragment corresponding to mRNA transcribed from the transgene (IGF1 and vector sequence) and a 200-bp band corresponding to endogenous IGF1. The mouse actin probe detected several bands resulting from cleavage by the RNase of mismatches between the mouse probe and the sheep actin mRNA. The numbers above the lanes are the sheep tag numbers. C is non-transgenic sheep skin RNA control. Y is yeast RNA.

Gene constructs. A recombinant wool follicle promoter-growth factor DNA construct was made by ligating a sheep IGF1 cDNA downstream from the mouse ultra-high-sulfur keratin gene promoter contained in the plasmid p2FOK[24]. The plasmid poIGF1[31], which contains a full-length ovine IGF1 cDNA cloned in pTZ18R-B, was digested with XbaI and the isolated insert cloned into XbaI digested p2FOK to yield the plasmid pKER-IGF (Fig. 1). For microinjection, pKER-IGF was purified by two rounds of cesium chloride centrifugation and the KER-IGF1 insert was released by digestion with KpnI and SacI then isolated from a 0.8% agarose gel by the glass-milk binding method (Geneclean, Bio101, La Jolla, California) according to the manufacturer's directions. The purified DNA was dissolved in TE buffer (10 mM Tris pH 7.5, 0.25 mM EDTA) at a concentration of 4 ng/μl and microinjected into pronuclear embryos as described by Wagner et al.[32].

Screening for transgene integration. Lambs born from microinjected embryos were tested for integration of the transgene by Southern blotting of DNA extracted from an ear biopsy taken shortly after birth, as described[33]. The entire ovine IGF-1 cDNA was random-prime labelled with ^{32}P (Amersham, Buckinghamshire; UK) and used to probe a Southern blot of BamHI digested sheep DNA. Transgenesis was assessed by the presence of a 700-bp band that is not detectable in non-transgenic sheep DNA. Because of cross-hybridization with the endogenous IGF1 gene, further analysis of integration was not possible.

Analysis of transgene expression. To provide a template for synthesis of a riboprobe to be used in the ribonuclease protection assay (RPA), pKER-IGF was digested with XbaI and the released cDNA fragment was treated with HphI to provide 200 bp of sheep IGF1 5′-DNA sequence plus 66 bp of pTZ18R-B vector sequence. This fragment was blunt-end cloned into the XbaI site of pBluescript SK (Stratagene, La Jolla, California) to give the plasmid pIGF-Δ-1. A radioactive antisense RNA was transcribed from pIGF-Δ-1 by T7 RNA polymerase in the presence of ^{32}P-UTP and used for RPA. The additional 87 bp of vector sequence in the riboprobe (66 bp from pTZ18R-B and 21 bp from pBluescript) allowed the transcript of the transgene to be distinguished from endogenous IGF1 mRNA.

Total RNA was extracted from sheep skin biopsies using Trizol™ solution (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. RPA was performed (Ambion, Austin, TX), by mixing 20 μg sheep skin total RNA with 10^5 cpm ^{32}P-riboprobe in hybridisation buffer (80% (v/v) formamide, 100 mM sodium citrate pH 6.4, 300 mM sodium acetate pH 6.4, 1 mM EDTA). A ^{32}P-labelled mouse actin antisense RNA was included to monitor uniformity of RNA concentration. The mixture was heated to 90°C to denature RNA, incubated

overnight at 45°C, then digested with 0.5 U RNase A and 20 U RNase T1 in 220 µl RNase digestion buffer (provided with the kit) for 30 min. at 37°C. After inactivation of RNase by incubation with the RNase inactivation buffer provided, the protected RNA fragments were precipitated according to the manufacturer's instructions, and analyzed by running in a 5% polyacrylamide gel in 0.09 M Tris, 0.09 M boric acid, 2 mM EDTA (TBE) at 200 V for 90 min. The gel was dried under vacuum and exposed overnight at −80°C to XAR X-ray film (Kodak, Rochester, NY).

Progeny testing. A transgenic ram expressing IGF1 mRNA in skin was mated to 43 random-bred non-transgenic ewes. The progeny were screened for inheritance of the transgene and run in separate mobs of males and females during the lamb year. Males were intact and the females were unmated. The animals were not sheared as lambs, thus shearing occurred as yearlings at approximately 14 months of age. A 10×10-cm mid-side wool patch was established in December 1993 and wool was clipped at 3-month intervals throughout the following year. At the same time, skin biopsies were taken for determination of IGF1 expression by RPA assay.

At yearling shearing, body weight was recorded, the fleece was weighed and a sample taken for scouring. Clean dry fleece weight, yield, fiber diameter, medullation, bulk, and staple strength were then assessed.

Wool measurement. To measure clean dry fleece weight, greasy wool samples were kept in a conditioned environment (20°C, 65% relative humidity (R.H.)) for 24 h, then washed in a sample wool scourer with nonionic detergent at 65°C, dried at 60°C for 20 minutes, kept in a conditioned environment at 20°C and 65% R.H. for 24 h then weighed. Yield was calculated as clean weight divided by greasy weight and expressed as a percentage. Fiber diameter was measured using an Optical Fibre Diameter Analyser (OFDA, DRAFT-TM-47-92, International Wool Textile Organisation, Ilkley, UK). Scoured wool was mini-cored into 2.0-mm snippets and sandwiched between two 72.0-mm square glass slides which were placed on the optical stage of the apparatus. The OFDA takes 2,000 measurements from the snippets and produces a mean fiber diameter figure. Four means were produced for each sheep and the mean of these was used as the final fiber diameter figure. To assess medullation, scoured staples were placed on a tray, covered with a solution containing benzyl alcohol with aniseed oil that has the same refractive index as wool, covered with glass, and examined visually. Each sample was measured twice and given a score ranging from 0 (no medullation) to 4 (heavy medullation). Bulk (the volume filling property of wool) was measured using a bulkometer (Wool Research Organisation of New Zealand, Lincoln, New Zealand). Scoured and carded full length wool was compressed by a piston in a cylinder to determine the bulk, expressed as cm^3/g. To measure staple strength, five sets of neighboring greasy staples were loaded in an Instron tensile strength instrument (Instron Ltd, UK). Staples were broken at a constant extension rate (100 mm/min) and staple strength was expressed as maximum load to break (Newtons/ktex), one kilotex being the density of a yarn weighing 1g/meter. Tests were carried out at 65% relative humidity and 20°C after preconditioning the samples to the environment for 24 h.

Statistical analysis. Data were analysed by the general linear model with body weight as the covariate, using the statistical package MINITAB[34] running on a personal computer. Statistical significance was chosen as a probability of less than 0.05.

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