DNA microsatellite analysis of Dolly

Dolly, the first animal cloned from an adult mammal, was produced by somatic cell nuclear transfer from a cell population derived from mammary tissue taken from a 6-year-old Finn Dorset ewe[1]. Analysis of DNA from Dolly showed that she contained the same seven microsatellite alleles as those present in the cell population from which she was derived[1]. Here we report a more detailed microsatellite analysis, which confirms the origin of Dolly.

Sgaramella and Zinder[2] recently asserted that sheep are "highly inbred", and they queried whether the microsatellite evidence that we originally provided[1] was sufficiently robust to exclude the possibility that Dolly was derived from embryonic or fetal cells from a different animal. They also suggested that, because the ewe was pregnant, Dolly might have been derived from a stray fetal cell contaminating the mammary tissue rather than from a mammary cell itself.

This hypothesis seems improbable as there are few fetal cells in the circulation of pregnant women (from 1 in 10^5 to 1 in 10^9 maternal cells[3]) and there is a much less intimate relationship between maternal and fetal circulation in sheep than in humans[4]. We think it highly unlikely that the partially purified mammary cells could have been overgrown by contaminating fetal cells during their relatively short time in culture[5].

Sheep populations are usually outbred rather than inbred and the five microsatellites used in the previous analysis (data from four of which were published[1]) were chosen because they are polymorphic in sheep[6]. However, in the absence of information about allele frequencies in the specific population from which the 6-year-old ewe was taken, we could not estimate how useful these markers are in discriminating between a fetal or maternal origin for Dolly. Thus we carried out the more detailed analysis described here.

The cells used to produce Dolly were prepared from mammary tissue taken from a 6-year-old ewe as part of a separate collaboration between the Hannah Research Institute in Scotland and PPL Therapeutics. A sample of the tissue that had been stored, frozen, at the Hannah Research Institute since the ewe was killed in 1995, and DNA from the original cell populations, was provided by C. Wilde for this analysis. We calculated the frequency of alleles in the Finn Dorset flock at the Hannah Research Institute from data from 44 individuals drawn from two separate generations but with only one representative from each full-sib family. Blood samples were delivered to Rosgen, a specialist genotyping company set up by the Roslin Institute and accredited

Table 1 **Microsatellite analysis of Dolly**			
Marker	Number of alleles found in Finn Dorset population	Alleles present in Dolly, mammary tissue and cultured cells	
		Size (base pairs)	Frequency in population (standard error)
TGLA53	7	151/151	0.55 (0.05)
SPS 115	4	248/248	0.22 (0.05)
TGLA126	7	118/126	0.17 (0.04)/0.24 (0.05)
TGLA122	9	190/190	0.07 (0.03)
ETH3	4	104/106	0.21 (0.04)/0.49 (0.05)
ETH225	4	148/150	0.62 (0.05)/0.02 (0.02)
FCB11	6	124/126	0.13 (0.04)/0.14 (0.04)
MAF209	4	109/121	0.17 (0.04)/0.57 (0.05)
FCB128	5	112/112	0.49 (0.06)
ETH10	1	208/208	1.00 (0.00)

to the quality standard ISO9002. DNA was extracted using standard protocols.

Microsatellite amplification was done using three of the primer sets used previously and seven proprietary markers from Perkin Elmer, and the products of DNA amplification by the polymerase chain reaction were analysed using an ABI 377 Prism Sequencer. The markers from Perkin Elmer were originally developed for parentage testing in cattle but had also been shown to be polymorphic in sheep. The presence or absence of each allele was determined in DNA extracted from blood from Dolly and the other Finn Dorset animals, from cells from the original mammary tissue, and from cells at passage four (Dolly was derived from cells at passage three).

The data on allele frequencies are summarized in Table 1. All but one of the ten microsatellite markers were polymorphic, with from four to nine alleles present per marker. The alleles present in DNA from Dolly were identical to those in the original mammary tissue, in the cell population prepared from that tissue and in the cells cultured to passage four. We estimated the probability that another sheep from the same population would have the same genotype as the 6-year-old ewe to be between 1.9×10^{-12} and 2.7×10^{-10} (95%

confidence interval). We conclude that it is extraordinarily unlikely that Dolly was derived from a different Finn Dorset animal and, therefore, reject the hypothesis that "imagined and unimagined experimental error"[2] occurred.

If Dolly were derived from a fetal cell, she would have derived half of her alleles from the sire of the fetus and half from the 6-year-old ewe. We calculated the chance of a fetal cell having the same genotype as the 6-year-old ewe to be between 1.1×10^{-6} and 9.2×10^{-6} (95% confidence interval). We conclude that Dolly was derived from a mammary cell of the 6-year-old donor ewe.

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DNA fingerprinting Dolly

The birth of Dolly[1] has raised considerable interest and debate over the potential for cloning mammals, including humans. However, there are concerns about the authenticity of Dolly, and whether she could have been derived not from an adult donor mammary cell, but instead from a contaminating sheep cell culture or from a fetal cell present in the udder of the pregnant ewe donor[2]. Microsatellite typing suggested but did not prove authenticity[1,2]. We

have therefore carried out a DNA fingerprint analysis to determine the origin of the donor cell used in nuclear transfer, and have confirmed the authenticity of Dolly.

The cell culture and micromanipulations used to make Dolly were done at PPL Therapeutics and the Roslin Institute, Edinburgh, using mammary cells prepared at the Hannah Research Institute from tissue of a late-pregnant Finn Dorset ewe[3]. Immunocytochemical analysis showed the

cell population to be predominantly (>98%) of epithelial origin, and able to express genes encoding milk proteins under permissive conditions. When cultured with lactogenic hormones on reconstituted basement membrane[4], the cells expressed messenger RNAs encoding α_{S1}-casein, β-casein and β-lactoglobulin. Serial passaging, as used for nuclear transfer[1], eliminated the cells' ability to express milk-protein genes.

We used DNA fingerprinting to compare cells from the original culture with mammary tissue from the donor ewe that had been stored frozen at the Hannah Institute and with a blood sample from Dolly provided by the Roslin Institute. Taking of the blood sample was witnessed.

As the sheep DNA fingerprinting systems described[5] are not highly discriminating, we used a cocktail of four different probes, each known to be effective at detecting variable minisatellites in various species[6,7] (our unpublished data). Southern blot hybridization with this cocktail (Fig. 1) showed that there is extensive variability in the DNA of Dolly and 12 control sheep, including a mother/offspring pair, from the Finn Dorset flock from which Dolly's donor was taken. We scored 58 different variable bands in total in these animals, with on average 17 such bands per sheep (range 13–22). Only 38% of the variable bands were shared between any two animals.

In contrast, the DNA fingerprints of the mammary tissue, the primary cell culture and Dolly were indistinguishable in terms of band number, position and relative intensity. Assuming a lack of linkage and association between different DNA fingerprint bands, as has been established for most bands in other mammalian multilocus analyses[8–10], the probability that a second unrelated sheep has, by chance, the same profile as the donor tissue can be conservatively estimated[9,10] at 6×10^{-10}. We therefore reject the possibility that Dolly was derived from a contaminating cell culture.

To determine whether Dolly could have been derived from a fetal cell, and could therefore be an offspring, rather than a clone, of the donor, we used band-sharing
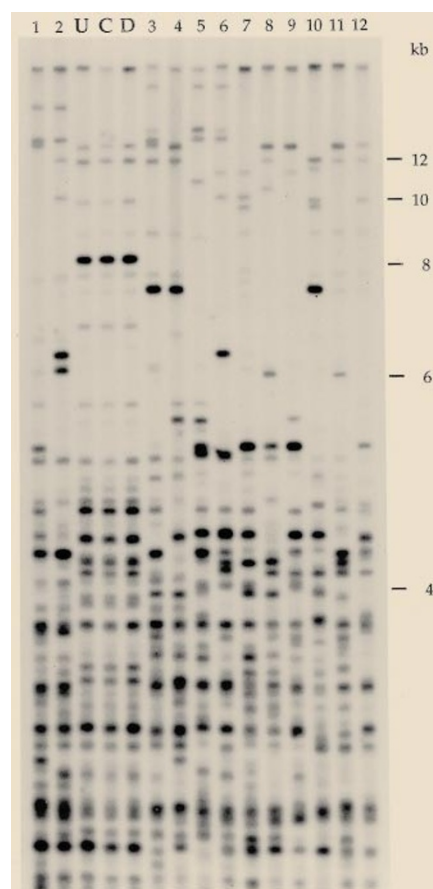

Figure 1 DNA fingerprint analysis of Dolly. DNAs were prepared from the donor udder (U), from the derived cell culture (C), and from blood from Dolly (D) and from control sheep 1–12. These DNAs were digested with *Mbo*I, electrophoresed through a 30-cm long 0.8% agarose gel, and Southern-blot hybridized[6] with a cocktail of ^{32}P-labelled probes comprising porcine S0322 (ref. 14), human MS1 (ref. 11), M13 phage DNA[7] and murine MMS10 (ref. 15). Samples 2 and 1 are from a ewe and her lamb, respectively. kb, kilobases.

data to calculate the probability that all 22 variable bands in the donor tissue would be present in an offspring of the donor[10]. This probability is low (8.6×10^{-5}) and is further reduced to about 3.5×10^{-7} by taking into account the lack of any paternal bands in this offspring that are not shared with the

donor tissue. Thus it is unlikely that Dolly was derived from a fetal cell.

To verify these findings, we retested all samples with a second cocktail of cloned human minisatellites MS40 (ref. 11), MS43 (ref. 11) and pλg3 (ref. 12) plus a $(GG A/T)_n$ repeat probe[13]. Highly variable profiles were obtained, with 14.8 ± 2.5 ($\pm$ s.d.) extra variable bands per sheep not detected by the first cocktail of probes. Again, Dolly, the mammary tissue and the cell culture were indistinguishable (data not shown). Inclusion of these additional polymorphisms in the statistical calculations above substantially lowered all probability values. We therefore conclude that Dolly is derived from the nucleus of a cell from the mammary gland of the adult donor.

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A cellulase gene of termite origin

The traditional view of cellulose digestion in animals is that they cannot produce their own cellulase, and so rely on gut microorganisms to hydrolyse cellulose. A classic example of this symbiosis is that between phylogenetically lower termites and the unicellular organisms (protists) that colonize their hindguts: cellulose fermented to acetate by the protists can be used as an

energy source by the termite[1]. There is evidence for the production of endogenous cellulase components by termites and other wood-feeding insects[2]; however, an unambiguous origin for such enzymes[1] has not been established, to our knowledge, until now. Here we describe the first insect cellulase-endoding gene to be identified, *RsEG*, which encodes an endo-β-1,4-glucanase (EC 3.2.1.4) in the termite *Reticulitermes speratus*.

Using antiserum raised against an endo-β-1,4-glucanase purified from *R. speratus*[3], we screened a recombinant phage comple-

mentary DNA library from this species and identified a partial sequence encoding a peptide with similarity to cellulases from glycosyl hydrolase family 9 (GHF9) (ref. 4). We then obtained the complete coding region of *RsEG* by rapid amplification of complementary DNA ends.

Although the source of messenger RNA for this study was the salivary glands, a part of termites that lacks microorganisms[2], we completely confirmed the endogenous origin of the gene by Southern blot analysis of DNA extracted from degutted termites (results not shown) and with amplification