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Red Deer Cloned from Antler Stem Cells and Their Differentiated Progeny[1]

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ABSTRACT

The significance of donor cell differentiation status for successful cloning by somatic cell nuclear transfer (SCNT) is unclear. Here, we cloned a new species, red deer *(Cervus elaphus)*, from multipotent antler stem cells and their differentiated progeny. Cultured donor cell lines from male antlerogenic periosteum (AP) were left undifferentiated or chemically induced to initiate osteogenesis or adipogenesis. Based on their morphology and marker gene expression profile, donor cells were classified as undifferentiated AP cells, presumptive osteoblasts, or adipocytes. Adipocytes upregulated adipogenic markers procollagen type I alpha 2 (*COL1A2*), peroxisome proliferator-activated receptor gamma 2 (*PPARG*), and gylceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and downregulated antlerogenic transcripts POU-domain class 5 transcription factor (*POU5F1*) and parathyroid hormone (*PTH*)-like hormone (*PTHLH*). Despite differences prior to NT, transcript abundance of donor-specific markers *COL1A2*, *PPARG*, *GAPDH,* and *POU5F1* did not differ significantly in cloned blastocysts ($P = 0.10$, 0.50, 0.61, and 0.16, respectively). However, donor cell and blastocyst expression levels were completely different for most genes analyzed, indicating their successful reprogramming. The type of donor cell used for NT (AP, bone, and fat cells), had no effect on in vitro development to blastocysts (93 [38%] of 248 vs. 32 [44%] of 73 vs. 59 [32%] of 183, respectively). Likewise, development to weaning was not significantly different between the three cell types (2 [4%] of 46 vs. 2 [29%] of 7 vs. 4 [13%] of 31, for AP vs. bone vs. fat, respectively). Microsatellite DNA analysis confirmed that the eight cloned red deer calves were genetically identical to the cells used for NT.

assisted reproductive technology, developmental biology, early development, embryo, pregnancy

INTRODUCTION

Since 1996, cloned offspring have been produced by somatic cell nuclear transfer (SCNT) in 15 mammalian species; namely, in chronologic order of publication: sheep [1], mouse [2], cattle [3], goat [4], pig [5–7], gaur [8], mouflon [9], domestic cat [10], rabbit [11], horse [12], mule [13], rat [14], African wildcat [15], dog [16], and ferret [17]. This ever-growing list of cloned species should not obscure the fact that SCNT remains very inefficient compared with other assisted reproductive technologies, such as in vitro fertilization (IVF) or artificial insemination. Typically, only 1%–5% of all cloned embryos transferred into surrogate mothers develop into viable offspring [18]. A number of approaches have been shown to improve in vitro development or gene expression, including better sources of recipient oocytes [17, 19, 20]; altering epigenetic marks in donor cells [21–23]; using chromatin transfer [24], serial NT [25], or sperm-mediated activation [26]; or aggregating somatic NT embryos [27]. However, significantly improved in vivo development has not been conclusively demonstrated for any of these treatments.

The first step of the cloning procedure is choosing a suitable nuclear donor cell type and cell cycle stage [28]. We and others have postulated that mammalian cloning efficiency is inversely correlated with the donor cell differentiation status and may be increased by using undifferentiated somatic stem cells as donors [28–30]. This hypothesis is mainly based on evidence from comparative mouse cloning experiments using 1) blastomere donor nuclei from early cleavage stages [20, 31, 32], 2) embryonic stem cells [33–35], and 3) terminally differentiated somatic donor cells, such as lymphocytes [36, 37] and neurons [2, 38–41]. These comparisons have demonstrated higher cloning efficiency with early blastomeres than with somatic cells [20]; however, they failed to conclusively determine whether differentiation status significantly affects cloning efficiency within somatic donor cell lineages [42].

In this study we have compared phenotypically distinct but genetically identical donors within the same somatic lineage in order to conclusively correlate mammalian cloning efficiency with differentiation status. We chose the antlerogenic periosteum (AP) lineage from red deer *(Cervus elaphus)* as a model system. Antlers are cranial appendages capable of repeated rounds of annual regeneration and are restricted to members of the deer family [43]. They develop postnatally from *pedicles*, a pair of permanent extensions of the frontal bone. Antlers comprise a number of different tissue types, including skin, blood, nerve, cartilage, bone, and connective tissue cells. Both pedicles and antlers derive from AP cells [44]. The cells are anatomically and histologically well defined, overlaying the two frontal crests of prepubertal deer frontal bone [45], and can be reproducibly sampled using standard biopsy techniques [46]. They display the defining characteristics of undifferentiated multipotent somatic stem cells; namely, the capability of lifelong self-renewal and production of differentiating daughter cells. In vivo, AP cells are responsible for the annual rounds of antler regeneration, giving rise to all different antler lineages (e.g., skin, blood, nerve, cartilage, bone, and connective tissue), even after ectopic transplantation into deer [44, 47] or the skull bone of nude mice [48, 49]. Their removal from the presumptive antler growth region abolishes future antler formation [47]. In vitro, AP cells are highly proliferative, giving rise to cell lines that can be differentiated into several mesodermal cell lineages, such as bone and cartilage and other

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types of connective tissue cells [49, 50]. These characteristics make AP cells a unique model system to study mammalian regeneration [43, 49] and an attractive candidate lineage to test the hypothesis that donor differentiation and cloning efficiency are inversely correlated.

MATERIALS AND METHODS

Chemicals were supplied by Sigma-Aldrich (Auckland, New Zealand), and all embryo manipulations were carried out at 38.5°C unless indicated otherwise. Investigations were conducted in accordance with the regulations of the New Zealand Animal Welfare Act 1999 and were approved by the Ruakura Animal Ethics Committee.

Isolation of Nuclear Donor Cells

Undifferentiated donor cells were isolated from the initial antlerogenic periosteum (IAP, or AP for short) of 4- to 8-mo-old male red deer heads that were collected from the local abattoir on two separate experimental occasions (designated stags 1 and 2), as described [46]. Briefly, the frontal crest area was thoroughly shaved and cleaned. A crescent-shaped incision was made on the scalp skin 2 cm medial to the frontal crest using a scalpel. The scalp skin was separated from the frontal bone and reflected laterally to expose the AP. Subcutaneous loose connective tissue was trimmed away. Following a scalpel incision on the periosteum, the exposed AP was peeled off from the underlying bone using a pair of rat-toothed forceps. Compared with facial periosteum, AP is denser and more than three times as thick [51], and can readily be peeled off. The AP sample was placed in Dulbecco modified Eagle medium (DMEM; Invitrogen, Auckland, New Zealand), with 10% fetal calf serum (FCS; Invitrogen), 2 mM GlutamaxII (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (AP medium), and it was transferred to the tissue culture laboratory. Cell culture was carried out as previously reported [52]. Briefly, AP pieces (about 1 mm^3) were transferred into a 50-ml centrifuge tube containing 20 ml DMEM, 2.5% FCS, and 200 U/ml collagenase (Invitrogen), and were incubated at 37°C for 3 h with the tube rotating at 100 rpm. After digestion, the cells were washed twice in AP medium and cultured on a T75 culture flask in AP medium in a humidified incubator (5% CO_2 in air) at 37°C. Upon reaching confluence after 4–5 days, the cells were trypsinized, frozen at 1×10^6cells/ml in DMEM, 15% FCS, and 15% of a mixture dimethyl sulphoxide:glycerol (4:6), and stored in liquid nitrogen.

In Vitro Differentiation of Nuclear Donor Cells

Prior to NT, cells were thawed and seeded at $2.5 \times 10^4/cm^2$. After 17–20 h, they were washed three times with PBS and serum starved for 4 days in DMEM/F12 (AP/F12 medium) containing 0.5% FCS with or without differentiation-inducing reagents. For the pilot AP vs. bone cell comparison, AP cells from stag 1 (passage 3) were either cultured in AP/F12 medium containing 0.5% FCS for 4 days (AP1) or chemically induced to initiate osteogenesis by supplementing the AP/F12 medium with 0.1 μM dexamethasone, 50 μM ascorbate, and 10 mM β-glycerophosphate for the last 2 days in vitro. For the AP vs. fat cell comparison, AP cells from stag 2 (passage 5) were either cultured in AP/F12 medium containing 10% FCS for 4 days, followed by serum starvation in 0.5% FCS for 4 days (AP2), or were chemically induced to initiate adipogenesis by supplementing the AP/F12 medium with 0.1 μM dexamethasone, 2.07 μM insulin, 0.45 mM isobutyl-methylxanthine, and 15% rabbit serum (Invitrogen) for the first 4 days in vitro, followed by serum starvation in adipogenic medium with 0.5% rabbit serum for 4 days. To assess morphology, cells were fixed in freshly prepared 4% (w/v) paraformaldehyde/4% (w/v) sucrose solution in PBS, pH 7.4, for 15 min at room temperature and were washed once in PBS and once in double-distilled H_2O before mounting (DAKO mounting medium; S3023; Med-Bio Ltd., Christchurch, New Zealand).

BrdU Labeling and Detection

DNA replication was assessed using 5-Bromo-2-deoxyuridine (BrdU) incorporation. Cells were cultured on glass coverslips and labeled in culture medium containing 10 μM BrdU for 22–24 h. Cells were washed one time in PBS, fixed in 2 ml ice-cold 50 mM ethanol-glycine buffer pH 2.0 (1 M glycine solution in H_2O diluted in absolute ethanol) for at least 20 min at −20°C, washed three times in PBS, and the was DNA hydrolyzed in 2 N HCl for 20 min at 38.5°C. After three PBS washes, cells were blocked, stained (using anti-BrdU IgG; 1:2000; Sigma B-2531), mounted, and quantified using an epifluorescence microscope (AX-70; Olympus) equipped with a Spot RT-KE slider CCD camera (Diagnostics Instruments Inc.).

Oil Red O staining

Cells were fixed in 4% paraformaldehyde/4% sucrose solution in PBS for 15 min at room temperature, washed three times in PBS, incubated for 15 min in 60% isopropanol, stained for 15 min in a freshly filtered solution of three parts saturated Oil red O (2 g in 500 ml isopropanol) and two parts ddH_20, rinsed briefly in 60% isopropanol, washed thoroughly in ddH_20, and finally mounted in DAKO mounting medium.

Total RNA Extraction

For each donor cell type, the expression of the following transcripts was measured by quantitative real-time RT-PCR in donor cells and NT blastocysts from each of the five NT runs: *18S* rRNA, gylceraldehyde-3-phosphate dehydrogenase (*GAPDH*), peroxisome proliferator-activated receptor γ 2 (*PPARG*), procollagen type I α2 (*COL1A2*), POU-domain class 5 transcription factor (*POU5F1,* also known as *OCT4*), parathyroid hormone (*PTH*)-like hormone (*PTHLH,* also known as *PTH*-related peptide or *PTHrP*), and *PTH*/*PTHLH* receptor type 1 (*PTHR1*; Table 1). The following samples were separately lysed in Trizol (Invitrogen): approximately 100 trypsinized AP cells (50 μl Trizol) and 3×10^4 adherent AP cells (250 or 500 μl Trizol) from NT run 1; 3×10^4 adherent AP cells from NT run 2; 3×10^4 adherent AP and fat cells from NT runs 3, 4, and 5. Pools of AP- and fat-derived D8 cloned blastocysts from NT runs 3 (five for AP: two grade 2, two grade 3, and one grade 4; and 11 for fat: four grade 1, three grade 2, and four grade 3), 4 (11 grade 3 for AP and 12 grade 3 for fat), and 5 (six grade 3 for AP and five grade 3 for fat) were lysed in 50 μl Trizol. Prior to chloroform extraction, 200 ng MS2 carrier RNA and 5 pg rabbit α-globin mRNA were added to each embryo sample as an exogenous standard. RNA was precipitated with isopropanol in the presence of 10 μg linear acrylamide, pelleted by centrifugation at $13\,000 \times g$ for 20 min, washed once with 70% ethanol, and dissolved in 8 μl DEPC-treated water. Genomic DNA was removed through digestion with 2 Kunitz units of amplification–grade DNase 1 (Invitrogen) for 1 h at 37°C, followed by heat inactivation of the enzyme for 10 min at 65°C. RNA was precipitated with ethanol, pelleted by centrifugation at $13\,000 \times g$ for 20 min, washed once with 70% ethanol, and dissolved in 11 μl DEPC-treated water.

Reverse Transcription (RT)

First-strand cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen) according to the manufacturer's instructions. All RNA of each sample was reverse transcribed only once, resulting in a single batch of cDNA per sample. Briefly, 1 μl random hexamers (50 ng/μl) and 1 μl of 10 mM dNTP mix were added to 11 μl total RNA sample and incubated for 5 min at 65°C, followed by immediate quenching on ice for 1 min. Reverse transcription was performed by the addition of 10 μl master mix containing 2 μl 10× RT buffer, 4 μl of 25 mM $MgCl_2$, 2 μl of 0.1 M DTT, 1 μl RNaseOUT (40 U/μl), and 1 μl SuperScript III reverse transcriptase (200 U/μl). The reaction mixture was first incubated for 10 min at 25°C, then for 50 min at 50°C, and then terminated by heating to 85°C for 5 min. Finally, 0.5 μl *Escherichia coli* RNase H (20 U/μl) was added and incubated for 20 min at 37°C before storage at −80°C and use for PCR. To determine the presence of contaminating genomic DNA, reverse transcriptase was omitted in one sample each time a batch of samples was processed for cDNA synthesis (designated −RT control).

Real-Time RT-PCR

The LightCycler system (Roche Diagnostics, New Zealand Ltd.) was used for PCR amplification and data analysis (software version 4.0). All reactions were performed with the LightCycler FastStart DNA Master^{PLUS} SYBR Green I Kit. Primers were either designed using LightCycler Probe Design 2.0 or were published previously (Table 1). For each primer combination, $MgCl_2$ concentration and annealing temperature were experimentally optimized on an MJ thermal cycler, MPTC-200. The ready-to-use "Hot Start" LightCycler reaction mix consisted of 0.4 μl of each primer (10 μM), 2.0 μl LightCycler SYBR Green I master mix, 5.2 μl DEPC water, and 2.0 μl cDNA template, adding up to a total volume of 10 μl loaded per chilled capillary. The following four-segment program was used: 1) denaturation (10 min at 95°C); 2) amplification and quantification (10 sec at 95°C, 10 sec at 56°C [*18S,* α-globin, *GAPDH*], or 10 sec at 60°C [*PTHLH, PTHR1, GAPDH, COL1A2*] followed by 10 sec [*18S,* α-globin, *GAPDH, COL1A2*] or 20 sec [*POU5F1, PPARG, PTHLH, PTHR1*] at 72°C with a single fluorescent measurement repeated 45 times); 3) melting curve (95°C, then cooling to 65°C for 20 sec, heating at 0.1°C–0.2°C sec^{-1} to 95°C while continuously measuring fluorescence); and 4) cooling to 40°C. Product identity was confirmed by amplicon size with 1.5%

TABLE 1. Primers used for RT-PCR.

Gene	GenBank accession no.	Sequence (5′–3′)[a]	Location	Product size (bp)	Real-time PCR melting peak (°C)	Annealing temp. (°C)	Reference no.
18S rRNA	AF176811	F: GACTCATTGGCCCTGTAATTGGAATGAGTC	146–178	87	85	56	This paper
		R: GCTGCTGGCACCAGACTTG	214–232				
GAPDH	BC102589.1	F: GGCGTGAACCACGAGAAGTATAA	463–485	257	86	56	[53]
		R: CCCTCCACGATGCCAAACT	582–600				
PPARG	Y12420	F: CGCACTGGAATTAGATGACAGC	1388–1409	214	87	56	[54]
		R: CACAATCTGTCTGAGGTCTGTC	1580–1601				
COL1A2	AB008683	F: TGCTGGCCAACCATGCCTCT	3880–3899	120	83	60	[54]
		R: CGACATCATTGGATCCTTGCAG	3999–3978				
POU5F1	AF022987	F: GGTTCTCTTTGGAAAGGTGTTC	516–537	333	89	56	This paper
		R: TGGCGACGGTTGCAAAACCA	829–848				
PTHLH	AY328402	F: TTTACGGCGTCGGTTC	138–153	341	88	60	This paper
		R: CGGAGATGTCACATAGGT	478–461				
PTHR1	AY328401	F: CAGCGAGTGCGTCAAG	511–526	200	88	60	This paper
		R: CGCGAAGCATGAAGGA	710–695				

[a] F, forward; R, reverse.

agarose gel electrophoresis after real-time RT-PCR and melting curve analysis (melting point peak 84°C for *18S* and *COL1A2*; 86°C for *GAPDH*; 88°C for *PPARG*; 89°C for *POU5F1, PTHR1,* and *PTHLH*; 92°C for α-globin; and about 81°C for primer-dimers).

Quantification of Gene Expression

External standard curves were generated from serial dilutions of bovine fibroblast cDNA containing the relevant target gene (10^0 to 10^{-4} in 1:10 steps, each point in duplicate) and one curve of high quality (reaction efficiency: 1.985 for *18S,* 1.989 for *POU5F1,* 2.195 for *GAPDH,* 1.991 for *PPARG*, 1.930 for *PTHR1*, 2.075 for *PTHLH*, and 2.191 for *COL1A2*) was saved for each target gene and imported for relative quantification for all experiments with the same parameters and conditions. Relative expression was calculated as described [55] by dividing absolute concentrations (in relative units) from the standard curve for [*target*] by [*18S*] for each sample. It has been shown that *18S* is a valid normalization scalar for serum-starved vs. nonstarved cells [56]. Only amplifications that fulfilled the following criteria were included in the analysis: 1) a single band of the expected size and/or a single melting peak at the expected temperature; 2) controls with H_2O or sample RNA not reverse transcribed (−RT) as templates had much higher Cp values and different melting peaks, indicating absence of contaminations, nonspecific product, or primer-dimers.

In Vitro Maturation of Oocytes (IVM)

In vitro–matured nonactivated metaphase II (MII)–arrested oocytes were derived as follows. Briefly, slaughterhouse ovaries were collected from mature cycling hinds, placed into saline (30°C) supplemented with 50 μg gentamycin, and transported into the laboratory within 4 h. Cumulus-oocyte complexes (COCs) were collected in HEPES-buffered medium 199 (H199; catalog no. 31100–035; Life Technologies) containing 15 mM HEPES, 5 mM $NaHCO_3$, and 0.086 mM kanamycin monosulfate, with 925 IU/ml heparin (Artex Ltd., Waipukurau, New Zealand) and 20 μl/ml 20% (w/v) albumin concentrate (Immuno-Chemical Products [ICPbio], Auckland, New Zealand) by aspirating 3- to 7-mm follicles into a 15-ml Falcon tube using a 20-gauge needle and negative pressure (32–35 mm Hg). Only COCs with a compact, nonatretic cumulus oophorus-corona radiata and a homogenous ooplasm were selected for IVM. COCs were washed twice in H199 with 10% (v/v) FCS (H199–10) and once in bicarbonate-buffered medium M199 with 25 mM $NaHCO_3$, 0.2 mM pyruvate, 0.086 mM kanamycin monosulfate, and 10% (v/v) FCS (B199–10). Seven COCs in 10 μl B199–10 were transferred into a 40-μl drop of IVM medium B199–10 with 10 μg/ml ovine FSH (Ovagen; ICPbio), 1 μg/ml ovine LH (ICPbio), 1 μg/ml 17-β-estradiol, and 0.1 mM cysteamine in 6-cm dishes (Falcon 35–1007; Becton Dickinson Labware, Lincoln Park, NJ) overlaid with paraffin oil (Squibb, Princeton, NJ). Dishes were cultured in humidified 5% CO_2 in air atmosphere. After IVM for 18–20 h, the cumulus-corona was dispersed by vortexing up to 180 oocytes in 500 μl of 1 mg/ml bovine testicular hyaluronidase in HEPES-buffered deer synthetic oviduct fluid (H-DSOF; 100 mM NaCl, 6.0 mM KCl, 0.6 mM $MgSO_4 \times 7H_2O$, 0.069 mM kanamycin monosulfate, 20 mM HEPES, 5 mM $NaHCO_3$, 0.33 mM pyruvate, 3.0 mM L-lactic acid hemicalcium salt, 1 mM glutamine, Eagle's basal medium essential amino acids 50×, MEM nonessential amino acids 100×, and 3 mg/ml fatty acid–free bovine albumin [ABIVP; ICPbio]), followed by three washes in H-DSOF containing 0.1 mg/ml cold soluble polyvinyl acetate (10–30 kDa).

NT and Artificial Activation

Five independent zona-intact SCNT experiments were performed essentially as described previously [57], with pilot NT runs 1 and 2 using AP1 and putative bone cells and NT runs 3, 4, and 5 using AP2 and fat cells.

Oocytes that had extruded a first polar body after 19–20 h in IVM were stained in H199–10, 5 μg/ml Hoechst 33342, and 7.5 μg/ml cytochalasin B (CB) for 5 min. Enucleation was carried out 20–22 h after maturation (hpm) in H199–10 with CB. Cytoplasts were washed extensively in H199–10 without CB and were held there until donor cell injection. A single-cell suspension of donor cells was prepared by trypsinization, centrifugation, and resuspension in H199 + 0.5% FCS containing CB, which also served as the holding medium until injection. Recipient cytoplasts were dehydrated in H199–10 and 5% sucrose, injected with a single donor cell, and rehydrated in two steps; first in H199–10 and 2.5% sucrose for 5 min and then in H199–10. Couplets were electrically fused at 24 hpm (range: 21.5–25.5 hpm) in buffer comprising 0.3 M mannitol, 50 μM $CaCl_2$, 100 μM $MgCl_2$, 500 μM HEPES, and 0.05% ABIVP, pH 7.3. Fusion was performed at room temperature using two DC pulses of 2.25 kV/cm for 15 μsec each, delivered by an ECM 200 (BTX, San Diego, CA). Following the electrical stimulus, the reconstructed embryos were washed in H-DSOF + 10% FCS and checked for fusion by microscopic examination 30 min later. Reconstructed SCNT embryos were artificially activated 3.5 h (range: 3–5.5 h) after fusion, using a combination of 5 μM ionomycin in HEPES-buffered IVF-DSOF [58] for 5 min and 2 mM 6-dimethylaminopurine in early DSOF for 3 h. Following three washes in H-DSOF, SCNT reconstructs were placed into in vitro culture (IVC).

In Vitro Culture

Ten reconstructed embryos were cultured in vitro for 7 days (Day 0 [D0] = fusion) in 20 μl biphasic DSOF, early and late [58], modified to contain 100 mM NaCl, 5.0 mM KCl, 0.7 mM $MgSO_4 \times 7H_2O$, 1 mM $NaSO_4$, 25 mM $NaHCO_3$, 0.069 mM kanamycin monosulfate, 3.0 mM L-lactic acid hemicalcium salt, 0.33 mM pyruvate, 0.1 mM glucose, 1 mM glutamine, Eagles basal medium essential amino acids 50×, MEM nonessential amino acids 100×, and 1 mg/ml ABIVP. On D4, embryos were changed into fresh late DSOF drops (early DSOF with the following modifications: 0.2 mM glucose, 0.15 mM NaH_2PO_4, 1.5 mM $CaCl_2 \times 2H_2O$, 5 mM Na lactate). All cultures were overlaid with mineral oil and kept in a humidified modular incubation chamber (ICN Biomedicals Inc., Aurora, OH) gassed with 5% CO_2, 7% O_2, and 88% N_2.

Embryo Transfer, Pregnancy Monitoring, and Controlled Calving

Total embryo development to blastocyst stage was assessed on D7 and grades 1 to 3 ($B^{1–3}$) blastocysts [59] were selected for embryo transfer (ET). Recipient hinds were synchronized as described [60]. For NT, three to five recipients were in two different locations, Ruakura (N = 16) and Invermay (N = 54). On D7, following the estimated time of estrus [61] (estrus = D0 = day of NT), a single morphologic grade 1 to 3 [59] cloned blastocyst ($B^{1–3}$) in Emcare

TABLE 2. Microsatellite genotyping of deer DNA.

Microsatellite DNA markers[a,b]												
Sample	1	2	3	4	5	6	7	8	9	10	11	12
AP1 donors	266/266	243/245	213/212	125/125	111/118	172/172	94/102	217/226	184/186	155/167	56/68	120/135
Clone 1	266/266	243/245	213/212	125/125	111/118	172/172	94/102	217/226	184/186	155/167	56/68	120/135
Clone 2	266/266	243/245	213/212	125/125	111/118	172/172	94/102	217/226	184/186	155/167	56/68	120/135
Clone 3	266/266	243/245	213/212	125/125	111/118	172/172	94/102	217/226	184/186	155/167	56/68	120/135
Control 1	**258/266**	**243/243**	**213/213**	**133/133**	**111/112**	**168/177**	**93/102**	**217/224**	**148/184**	**155/155**	**56/61**	**135/135**
Control 2	**267/267**	**243/243**	**225/213**	**129/133**	**107/111**	**172/175**	**94/98**	**222/226**	**180/194**	**155/164**	**63/68**	**133/140**
AP2 donors	265/265	243/243	213/209	125/125	164/164	105/105	217/217	217/217	182/184	155/182	61/76	120/140
Clone 4	265/265	243/243	213/209	125/125	164/164	105/105	217/217	217/217	182/184	155/182	61/76	120/140
Clone 5	265/265	243/243	213/209	125/125	164/164	105/105	217/217	217/217	182/184	155/182	61/76	120/140
Clone 6	265/265	243/243	213/209	125/125	164/164	105/105	217/217	217/217	182/184	155/182	61/76	120/140
Clone 7	265/265	243/243	213/209	125/125	164/164	105/105	217/217	217/217	182/184	155/182	61/76	120/140
Clone 8	265/265	243/243	213/209	125/125	164/164	105/105	217/217	217/217	182/184	155/182	61/76	120/140
Control 3	**266/266**	243/243	**221/209**	125/125	**177/177**	**96/105**	**208/217**	217/217	**180/192**	**155/157**	**61/63**	**127/133**
Control 4	**266/266**	**243/247**	**219/209**	125/125	**174/174**	**98/109**	217/217	**221/226**	**180/180**	**155/155**	61/70	**127/146**

[a] The numbers indicate the size of each allele in base pairs.
[b] Bold text indicates informative markers distinguishing somatic cell-derived clones from genetically unrelated control animals.

embryo holding solution (ICPbio) was loaded per 0.25-ml straw (Cryo-Vet, Quebriac, France) and transferred nonsurgically into the uterine lumen ipsilateral to the corpus luteum. Embryos transferred at Invermay were held in straws for 7–12 h, whereas embryos transferred at Ruakura were held 3–5 h before transfer. Using ultrasonography (Aloka SSD-500 scanner with a 5-MHz linear rectal probe; Aloka Co. Ltd., Tokyo, Japan) the pregnancy status of recipient hinds was determined on D35 of gestation. Development throughout gestation was monitored approximately every 30 days, from D35 to D90 by rectal ultrasonography and thereafter to D190 transabdominally using a 3.5-MHz convex array (Aloka 500) probe placed against the abdominal wall cranial to the mammary gland. Mammary development was monitored weekly from D215 to D230, whereas hinds were visually monitored daily from D220 and then thrice daily beginning at D228. Milk appearing from the mammary gland was the criterion for allowing hinds to calve naturally following normal animal husbandry methods, including rearing their offspring. Manual assistance was only provided when hinds were in distress. Calves were weighed, tagged, and blood sampled within 24 h of birth. Calves were weaned when they were 4 mo of age.

DNA Microsatellite Analysis

Using standard protocols, DNA was extracted from AP donor cells and jugular blood samples of control calves and cloned offspring. Double-blind analysis of 12 proprietary microsatellite sequences (Table 2) was done independently by GenomNZ (Mosgiel). Length variations were assayed by PCR with fluorescently labeled locus-specific primers (forward primer labeled as follows: markers 1, 5, 7, and 12 with NED; 2, 3, 6, and 8 with FAM-6; 4 and 10 with PET; and 9 and 11 with VIC). The PCR products were run on an automated DNA sequencer (3730 DNA Analyzer; Applied Biosystems), and product size was estimated using Genemapper v3.7 software (Applied Biosystems).

Statistical Analysis

All values are presented as mean ± SEM. Statistical significance was determined using the two-tailed *t*-test with equal variance for gene expression data or the two-tailed Fisher exact test for independence in 2 × 2 tables for development data. For microsatellite analysis, the probability of a match from a randomly selected individual was calculated as the profile probability, assuming that loci were in linkage and Hardy-Weinberg equilibrium [62]. Allele frequencies were estimated from a standard set of red deer.

RESULTS

In Vitro Differentiation of AP Cells

All AP1 cells responded rapidly (within 24 h) to the bone differentiation medium by a drastic change in morphology (Fig. 1). While AP1 cells were typically bipolar or multipolar with their length more than twice their width ("fibroblast-

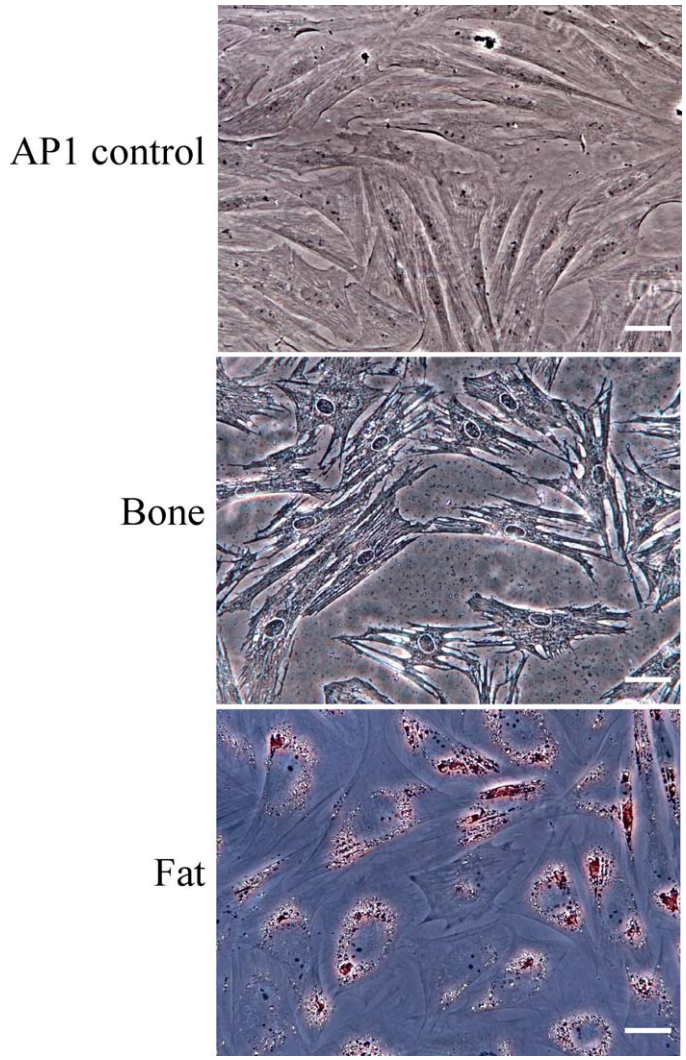

FIG. 1. Morphology of donor cells for red deer SCNT. Phase-contrast images of monolayer cultures of undifferentiated AP donor cells (top panel). AP1 cells after 2 days of induced bone differentiation (middle panel). AP2 cells after 4 days of induced fat differentiation in 15% serum (bottom panel). All cells were cultured in 0.5% serum for 4 days before fixation. Bar = 10 μm.

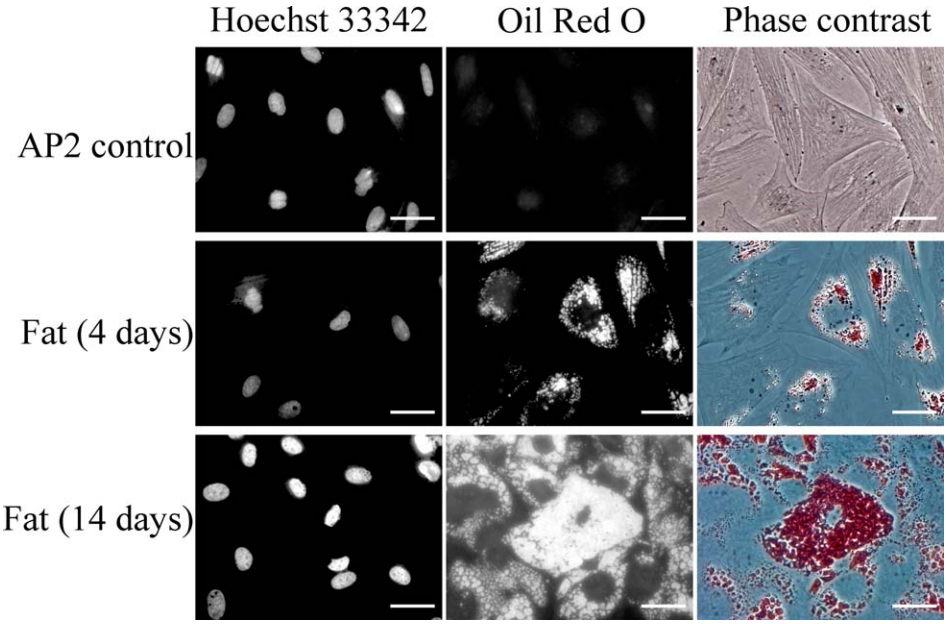

FIG. 2. Morphological differentiation of cervine AP cells vs. AP-derived adipocytes. AP2 cells were left undifferentiated (AP control) or chemically induced to initiate adipogenesis for 4 days (short term) or 2 wk (long term) in vitro. Cells were fixed, double stained for DNA (Hoechst 33342) and lipid accumulation (Oil Red O), and observed under epifluorescence (left and middle panels) and phase contrast (right panel). Bar = 10 μm.

like"), they changed to a very distinct appearance, featuring a large number of spiky projections and irregular filopodia-like protrusions at the periphery. We then attempted targeted differentiation of AP2 cells into adipocytes, a morphologically and molecularly well-defined donor cell type that they would not naturally form in vivo. Again, the cells responded by changing their fibroblast-like appearance to a wider, less elongated, and more polygonal morphology within 24 h (Fig. 1). After 4 days in fat differentiation medium (short term), more than 99% of all cells (322 [99.4%] of 324) had accumulated cytoplasmic lipid droplets that stained positive with Oil Red O (Fig. 2). Upon prolonged culture in fat medium for 2 wk (long term), the proportion of Oil Red O-positive cells (303 [100%] of 303) as well as number and size of lipid droplets per cell increased further, until they almost completely filled the cytoplasm (Fig. 2). None of these changes were observed in the serum-starved AP2 control cells. The morphological change correlated with changes in relative gene expression of early, intermediate, and late markers of adipogenesis (Fig. 3A) [63]. Their drastic change in cell shape was paralleled by a 2.5-fold upregulation of relative procollagen type I (*COL1A2*) expression (6.85 ± 1.18 to 16.93 ± 0.80; $P = 0.001$). Deposition of lipid stores was accompanied by the appearance of transcriptional regulator peroxisome proliferator-activated receptor γ-2 *(PPARG)* [63], which was upregulated almost 2 fold (0.86 ± 0.09 to 1.37 ± 0.13; $P = 0.03$). Members of the PPAR family of nuclear hormone receptors activate genes that are involved in triacylglycerol metabolism, such as *GAPDH* [64], which was upregulated more than 3-fold in our cultures (1.73 ± 0.25 to 5.38 ± 0.34; $P = 0.0003$). In addition, we also observed downregulation of two transcripts enriched in AP2 cells, *POU5F1* (also known as

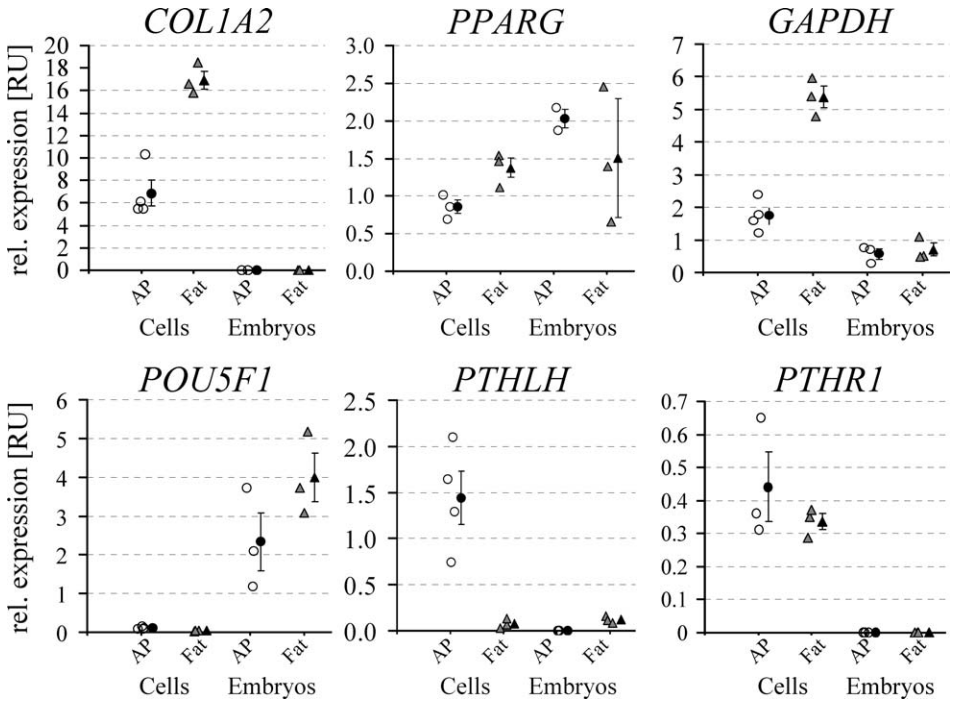

FIG. 3. Gene expression in cervine donor cells and cloned embryos. Quantification of target gene transcript abundance normalized on *18S* expression (relative units [RUs]) in AP2 cells vs. AP2-derived blastocysts and adipocytes vs. adipocyte-derived blastocysts (open circles vs. gray triangles, respectively). Each data point represents one cell sample or pool of blastocysts, respectively, from a different NT run. RNA was extracted and reverse transcribed on the same day for all cell and blastocyst samples. Each sample was measured at least twice for both target and reference genes. Arithmetic means for AP2 vs. adipocyte samples are shown as filled circles or triangles ± SEM, respectively.

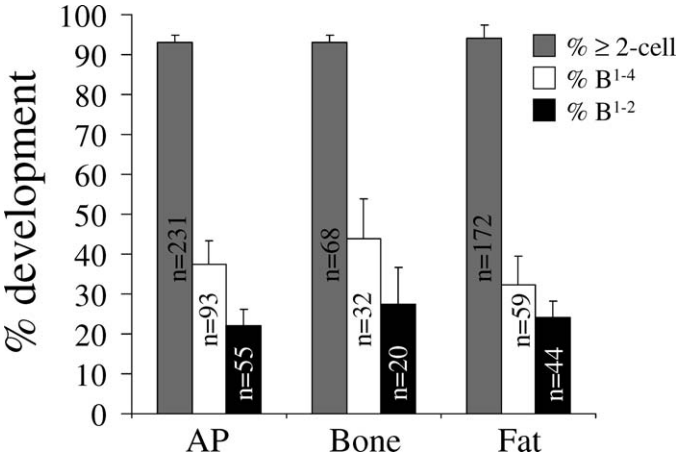

FIG. 4. In vitro development of AP-derived vs. bone-derived vs. fat cell-derived cloned cervine embryos. Quantification of cloned red deer embryo development following NT with AP, bone, or fat cells. % Development, the proportion of the total number of reconstructed embryos placed into culture that developed into two or more cells, blastocysts (B) grades 1–4 (B^{1-4}) or B^{1-2} (% ± SEM); n, total number of embryos that developed to this stage in five independent NT experiments (NT 1–2 with AP1 vs. bone, NT 3–5 with AP2 vs. fat).

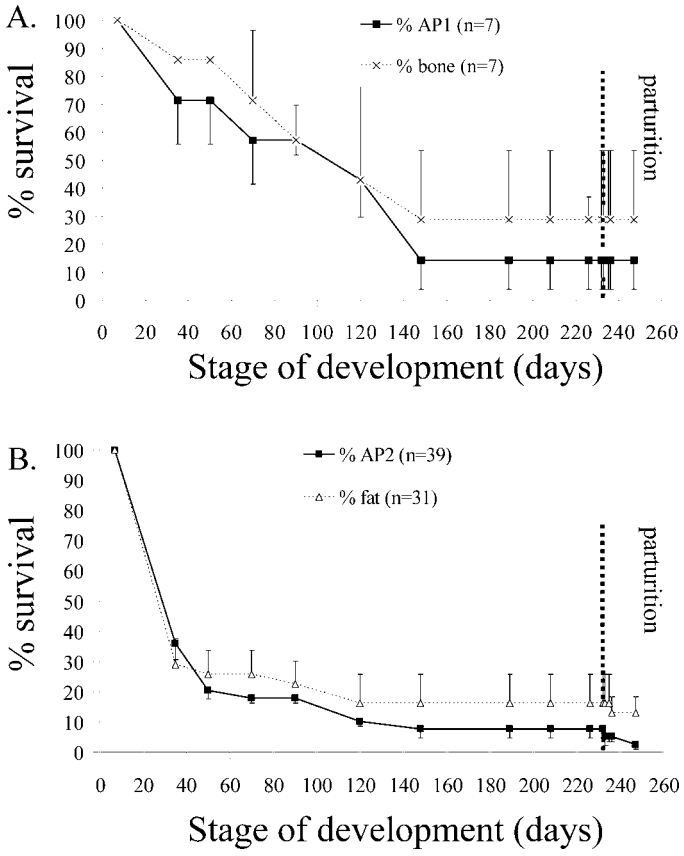

FIG. 5. In vivo development of AP-derived vs. bone-derived vs. fat cell-derived cloned cervine embryos. Clones were obtained after NT with (**A**) AP vs. AP-derived bone cells from stag 1 (AP1) and (**B**) AP vs. AP-derived fat cells from stag 2 (AP2). Stippled vertical line indicates the average onset of parturition. % Survival, proportion of total number of embryos transferred that developed to fetuses and calves (% ± SEM); n, total number of embryos transferred.

OCT4) and parathyroid hormone (*PTH*)-like hormone (*PTHLH*). We measured specific *POU5F1*-expression in AP2 cells which was 2.5-fold downregulated upon adipocyte differentiation (0.10 ± 0.02 to 0.04 ± 0.01, $P = 0.02$). Likewise, *PTHLH*, a marker of AP tissue in vivo [65], was 18-fold downregulated in adipocytes (1.44 ± 0.29 to 0.08 ± 0.03; $P = 0.01$), whereas *PTH/PTHLH* receptor (*PTHR1*), another AP marker, showed no change (0.44 ± 0.11 to 0.34 ± 0.03; $P = 0.39$). Serum starvation effectively induced quiescence across all cell types compared with nonstarved undifferentiated AP cells. After 24 h BrdU labeling, the proportion of BrdU-incorporating undifferentiated AP cells (expressed as a percentage of BrdU-positive per Hoechst 33342-positive nuclei) was significantly reduced from 81.9% (570 of 696) in culture medium with 10% FCS to 1.4% (13 of 955), 0% (0 of 234), and 1.8% (14 of 793) for undifferentiated AP, putative bone, and fat cells in culture medium containing 0.5% serum for 4 days, respectively ($P < 0.0001$).

Reprogramming of Donor Cell Expression after NT

To evaluate donor cell reprogramming following NT, relative transcript levels of donor cell marker genes were measured in pools of AP2-derived vs. adipocyte-derived blastocysts (Fig. 3). Despite differences prior to NT, transcript abundance of donor-specific markers *COL1A2*, *PPARG*, *GAPDH,* and *POU5F1* did not differ significantly in cloned blastocysts ($P = 0.10$, 0.50, 0.61, and 0.16, respectively; Fig. 2). However, donor cell and blastocyst expression levels were completely different for most genes analyzed (Fig. 3). This was most obvious for *COL1A2,* which was 1000- to 10 000-fold downregulated (6.85 vs. 0.001 ± 0.0003 and 16.93 vs. 0.0005 ± 0.00002; $P = 0.01$ and $P = 0.0007$, respectively), and *POU5F1,* which was 20- to 100-fold upregulated (0.10 vs. 2.33 ± 0.75 and 0.04 vs. 3.99 ± 0.62; $P = 0.02$ and $P = 0.0006$, respectively) in AP2 cells vs. AP2-derived blastocysts and adipocytes vs. adipocyte-derived blastocysts, respectively. *GAPDH* was 3- to 8-fold downregulated (1.73 vs. 0.56 ± 0.15 and 5.38 vs. 0.70 ± 0.20; $P = 0.01$ and $P = 0.02$, respectively), whereas *PTHR1* expression was silenced below measurable levels compared with the donor cells (0.44 vs. 0 and 0.34 vs. 0; $P = 0.01$ and $P = 0.007$, respectively). The only exceptions were *PPARG* and *PTHLH,* where adipocytes and adipocyte-derived blastocysts had very similar relative expression levels. However, both genes were reprogrammed in AP2-derived blastocysts (0.86 vs. 2.03 ± 0.12 and 1.44 vs. 0; $P = 0.006$ and $P = 0.008$, respectively).

Effect of Donor Cell Type on In Vitro Embryo Development

Polar body extrusion around 20 hpm was 1340 of 1718 (78% ± 6%), and only those oocytes were considered mature and selected for enucleation. SCNT efficiency, as measured by the proportion of successfully fused cytoplast-donor couplets out of all fusion attempts, clearly depended on the donor cell type and was highest using adipocytes (252 [59.2%] of 426 vs. 84 [73.7%] of 114 vs. 188 [84.3%] of 223 for AP, bone, and fat cells, respectively; $P < 0.01$ for AP vs. bone, $P < 0.0001$ for AP vs. fat, and $P < 0.05$ for bone vs. fat cells). Following IVC, we found no significant effect of differentiation status on development (Fig. 4). Comparing AP, bone, and fat cells, cleavage to two-cell (215 [93%] of 231 vs. 63 [93%] of 68 vs. 161 [94%] of 172, respectively), total development into grade 1–4 (93 [38%] of 248 vs. 32 [44%] of 73 vs. 59 [32%] of 183, respectively) and transferable grade 1–2 blastocysts (55 [22%] of 248 vs. 20 [27%] of 73 vs. 44 [24%] of 183, respectively) was very similar.

Effect of Donor Cell Type on In Vivo Embryo Development

Holding cloned embryos in straws for up to 12 h before transfer did not significantly affect pregnancy rate on D35 (NT3/Ruakura site: 7 [44%] of 16 vs. NT 4–5/Invermay site: 15 [28%] of 54; $P = 0.37$). Likewise, embryo survival into adulthood was not significantly different between the two recipient pools at Ruakura or Invermay. Therefore, results are presented as pooled data. Following embryo transfer, postblastocyst viability was not dependant on donor cell differentiation (Fig. 5). For the AP1 vs. bone cell comparison, overall survival rates to D35 and weaning were relatively high; however, there was no difference between embryos derived from AP1 or bone cells (AP1: 5 [71%] of 7 vs. 6 [86%] of 7; bone: 1 [14%] of 7 vs. 2 [29%] of 7). Likewise, for the AP2 vs. fat cell comparison, survival to D35 and weaning was very similar, albeit lower overall, between the two donor cell types (14 [36%] of 39 vs. 9 [29%] of 31; and 1 [3%] of 39 vs. 4 [13%] of 31, respectively). Red deer clones died between ET on D7 and the first scan on D35 (50 [60%] of 84), followed by a steady rate of attrition from D35 to D150 (23 [68%] of 34), and no further losses to term. Of the 11 calves born, 3 died before weaning (3 [27%] of 11). The first calf was stillborn as a result of dystocia and had no obvious abnormalities; the second calf died shortly after birth with a number of pathologies (contracted flexor tendons in all legs, enlarged fatty liver, and incomplete lung inflation); and the third was found dead 6 days after birth, apparently abandoned by its recipient dam, with no postmortem abnormalities. A similar situation occurred with one control calf, which was found dead 2 days after birth showing no abnormalities. A total of 11 male calves developed to term, 8 of which have survived beyond weaning (two from AP, two from putative bone, and four from adipocyte donors). The overall cloning efficiency across all cell types was 13% (11 of 84) to term and 10% (8 of 84) to weaning and beyond. Survival to weaning was not significantly different between AP, putative bone, and fat cells (2 [4%] of 46 vs. 2 [29%] of 7 vs. 4 [13%] of 31, respectively). Average birth weights (11.2 $\pm$ 1.1 vs. 9.9 $\pm$ 0.1 vs. 9.8 $\pm$ 1.1 kg, respectively) and gestation length (237 $\pm$ 3.3 vs. 233 $\pm$ 0.5 vs. 232 $\pm$ 2.1 days, respectively) did not differ between AP, bone, and fat cells, respectively. Overall, average birth weight (10.3 $\pm$ 0.6 kg) and gestation length (234 $\pm$ 1.6 days) were comparable to naturally mated male half-siblings (10.8 $\pm$ 0.6 kg and 234 $\pm$ 2.5 days, respectively). Microsatellite analysis of 12 proprietary sequences confirmed that all eight surviving clones were genetically indistinguishable from their respective donor cells. The probability of a random deer sharing this genetic profile is approximately 2×10^{-16}. Genetically unrelated control calves had multiple differences in all microsatellite sequences analyzed (Table 2). So far, all calves appear healthy and have survived beyond weaning (earliest born: March 2004; latest born: December 2005, Fig. 6).

DISCUSSION

We report the first cloned red deer in this paper, the 16th mammalian species to be successfully cloned. Most of the previous species were first cloned from either follicular cells or fibroblasts. Here we chose an altogether different source of donor cells that is specific to cervine species; namely, cells forming the antlerogenic periosteum. AP cells were chosen because their proliferation and differentiation potential makes them a suitable donor cell source to test the idea that donor cell differentiation, plasticity, and cloning efficiency are related. We found that for this particular lineage this was not the case.

AP was initially discovered in 1974 as the tissue that gives rise to antlers [44]. The cells are anatomically and histologically well defined and have demonstrated full antlerogenic potential by giving rise to pedicle-like or antler-like protuberances after transplantation at ectopic sites in deer [44, 47] and nude mice [48, 49]. They can also be readily differentiated into several mesodermal lineages in vitro [49]. In addition to their ability to differentiate in vitro into lineages that naturally occur in antler (such as bone and cartilage), we have demonstrated here that these cells can also be differentiated into adipocytes with near 100% efficiency. Taken together, AP cells display many defining characteristics of undifferentiated multipotent somatic stem cells; namely, the capability of lifelong self-renewal, proliferation, and the production of differentiating daughter cells. They share these characteristics with adult stem cells from the bone marrow, nervous system, skin, muscle, and intestine, which are also thought to be responsible for regenerating damage and maintaining tissue homeostasis [66].

We have compared phenotypically distinct but genetically identical donors within the AP somatic lineage; namely, undifferentiated AP cells vs. their in vitro-differentiated progeny in particular adipocytes. Several lines of evidence support that we have used distinctly different donor cells for NT. First, the AP, putative bone, and fat cells differed morphologically, with AP cells being fibroblast-like, bone cells having spiky projections characteristic for this cell type [67], and adipocytes being more polygonal and showing obvious accumulation of cytoplasmic lipid droplets that further increased over time (Fig. 1). Despite the clear morphologic differences, we were unable to detect molecular differences between undifferentiated AP1 control cells and AP1 cells cultured in bone differentiation medium for 2 days. Since the bone differentiation was only done for 2 days, there were no signs of mineralization using von Kossa stain to detect calcium deposits (data not shown). Likewise, immunocytochemistry against osteopontin and osteonectin revealed that both cell types were equally positive (data not shown). Using real-time RT-PCR, we did not detect significant differences between AP1 and putative bone cells for the following genes: *GAPDH*, *PPARG*, *COL1A2*, *POU5F1, PTHLH*, *PTHR1,* and osteopontin (data not shown). In our main cloning trial (NT3–5), we therefore focused on the AP2 vs. adipocyte comparison. AP2 cells and adipocytes differed in the expression level of a number of molecular markers. During in vitro adipogenesis, cells differentiate from multipotent, mesenchymal precursors or stem cells, which can give rise to muscle, cartilage, bone, or fat, into preadipocytes, which are operationally defined as transiently proliferating cells that have passed the critical transition point of commitment to the fat lineage. Preadipocytes express a number of early marker genes but are defined as being fibroblastic in shape and still lipid free [68]. After 4 days in fat differentiation medium, almost all cells had accumulated cytoplasmic lipid droplets, indicating that they had passed the preadipocyte stage and developed into terminally differentiated adipocytes (Fig. 2). Due to these lipid droplets, trypsinized donor cells had a very dark appearance that was readily visualized using phase-contrast and stereo microscopy and facilitated donor cell selection for SCNT. Adipocyte-derived SCNT embryos were also filled with lipid droplets and appeared a lot darker than control embryos under stereo microscopic observation. Terminal differentiation into mature adipocytes occurs through changes in gene expression whose exact hierarchy and chronology have not yet been fully determined [69, 70]. However, a number of early, intermediate, and late expression markers have emerged from these studies. The drastic changes in cell shape are paralleled by changes in

FIG. 6. Red deer cloned from antlerogenic stem cells. Male red deer clones from antler stem cells or stem cell-derived adipocytes, born November/December 2005, at 5 mo of age.

the level and type of components secreted into the extracellular matrix [63, 71]. Accordingly, we have observed a 2.5-fold upregulation of relative *COL1A2* expression. The process of terminal differentiation involves the deposition of lipid stores, accompanied by the appearance of transcriptional regulators, such as *PPARG*, which was upregulated almost 2-fold. Members of the PPAR family of nuclear hormone receptors are involved in activating genes that are involved in triacylglycerol metabolism, such as *GAPDH* [64, 72], which was upregulated more than 3-fold in differentiating cultures. In addition to the upregulation of adipocyte-specific genes, we also observed downregulation of two transcripts enriched in AP2 cells, *POU5F1* and *PTHLH*. *POU5F1*, for example, has usually been described as a marker of premeiotic germ cells, pluripotent embryonic cells, and their derivatives (embryonic stem cells and embryonic germ cells). Robust expression, however, has recently also been described in mesenchymal stem cells from bone marrow [73] and cultured skin-derived fetal fibroblasts [74, 75], suggesting that it may also be a marker for undifferentiated somatic stem cells. Specific *POU5F1* expression in AP2 cells was 2.5-fold downregulated upon adipocyte differentiation. Likewise, *PTHLH*, which has been found as a protein in AP tissue in vivo [65], was 18-fold downregulated in adipocytes. Based on this evidence, two very distinct cell types from the same lineage were used for NT: undifferentiated AP cells and mature adipocytes.

The mechanism by which AP cells gave rise to differentiated progeny is presently unclear. Fat differentiation was done in the presence of 0.5% rabbit serum. Under this condition, less than 2% of the population was proliferative, as measured by the incorporation of BrdU after a 24-h labeling period. In addition to the lack of DNA synthesis, Hoechst 33342 nuclear staining revealed that there were no mitotic cells present in the population of serum-starved AP or fat cells. Therefore, it is unlikely that differentiated cells arose by stem cell division, be it asymmetric (one stem cell divides into one differentiated daughter and another stem cell) or symmetric (one stem cell divides into two differentiated daughters). Given that more than 99% of all cells in the monolayer had acquired the distinct putative bone cell or adipocyte morphology, it is more likely that this was a result of a direct conversion, or transdifferentation, from the existing undifferentiated AP state into the differentiated phenotype. However, we have not performed any lineage-tracing experiments to demonstrate a direct ancestor-descendant relationship between the two cell types.

There is evidence that cloned embryos can retain the gene expression program of the donor cell to some degree [76, 77]. On the other hand, most of the donor cell-specific genes that we have studied had highly significantly different expression levels at the blastocyst stage. This was most obvious for *COL1A2,* which was 1000- to 10 000-fold downregulated, and *POU5F1*, a key embryonic gene, which was 20- to 100-fold upregulated in AP2 and adipocyte-derived blastocysts, respectively. *GAPDH* was 3- to 8-fold downregulated, whereas *PTHR1* expression was silenced below measurable levels compared with the donor cells. The only exceptions were *PPARG* and *PTHLH,* where adipocytes and adipocyte-derived blastocysts had very similar relative expression levels. Importantly, none of the significant expression differences between donor cells prior to NT were apparent at the blastocyst stage. This indicates that reprogramming of donor gene expression occurred similarly for both cell types and was independent of the expression status of a given gene at the time point of NT.

In keeping with the similar degree of gene expression reprogramming for AP2 cells vs. adipocytes, we also found no differences in development to the blastocyst stage. Across all three donor cell types, total development to grade 1–4 blastocysts (184 [37%] of 504) or grade 1–2 blastocysts (119 [24%] of 504) was only slightly lower than average results with bovine fibroblast donor cells in our hands (681 [48%] of 1420 and 481 [34%] of 1420, respectively; Oback, unpublished data). That the rate of blastocyst formation is not indicative of subsequent development has been shown many times before. For example, apparently normal blastocyst formation can be achieved after NT with primordial germ cells free of imprints [78, 79] or cancer cell nuclei harboring nonreprogrammable mutations [80, 81], both of which are conditions that will prevent development to term. Even in cases where closely related cell types differ significantly in blastocyst development—for example, lung vs. muscle fibroblasts [82] and different cell lines of adult ear skin fibroblasts [83] in cattle or natural killer vs. helper T cells in mouse [37]—this did not correlate with significant differences in cloning efficiency.

In the absence of other conclusive correlations, survival into adulthood is currently the most informative and meaningful measure of extensive donor cell reprogramming [42]. Importantly, we did not observe that postblastocyst viability depended on donor cell differentiation status. Survival to weaning was not significantly different between AP, presumptive bone, and fat cells (2 [4%] of 46 vs. 2 [29%] of 7 vs. 4 [13%] of 31, respectively). This lack of statistical significance may simply mean that the differences in cloning efficiency between donor cells were too subtle to be detectable with the number of replicate cloning experiments performed. Contrary to previous studies [84, 85], it may also indicate that the degree of donor cell differentiation is independent from its ability to be reprogrammed after NT.

The overall cloning efficiency across all cell types was 13% (11 of 84) to term and 10% (8 of 84) to weaning. This is comparable to our cattle cloning results at AgResearch, where 133 (13%) of 988 developed to term and 89 (9%) of 988 to weaning [86]. The survival profile of red deer clones was also similar to somatic cattle clones; however, there were no losses

in the last two thirds of gestation. This was associated with a notable absence of hydroallantois in the cloned pregnancies. In cattle, excess allantoic fluid accumulation during pregnancy is a major animal welfare issue. Depending on the donor cell line and cell cycle stage used for SCNT, hydroallantois can account for anywhere between 0 and 86% of all failing pregnancies between D120 to term [87]. All deer recipients developed functional mammary glands and initiated parturition signaling, negating the need for hormonal induction, which is the standard practice in our cattle cloning [86]. The 27% of losses between term and weaning were comparable to survival data in cattle, where in our hands about one third of cloned offspring die during this period [86].

The first cloning of red deer has implications for basic research, industry, and conservational biology. From a basic research perspective, access to large numbers of genetically identical, immunocompatible experimental animals will facilitate transplantation experiments and be of great interest to distinguish between genetic and epigenetic effects on antler regeneration, growth, and development [43]. It will also benefit research into desirable immunologic traits, such as tuberculosis (Tb) resistance in livestock. Expanding already existing red deer breeding lines, selected on the basis of their differing responses to experimental challenge with *Mycobacterium bovis*, through cloning would eliminate genetic variation in future microarray studies to identify markers of Tb resistance [88]. Such markers are likely to be similar in other domestic ruminants and humans, and their discovery would be of interest also outside the deer industry. The potential use of SCNT for conservation of endangered species has been first exemplified through the cloning of the last remaining cow of the Enderby Island cattle breed [89]. This was followed by production of cloned offspring from gaur cattle [8], mouflon sheep [9], and African wildcats [15]. Cloning efforts in a number of other threatened or endangered species, such as Argali sheep [90], Banteng [91], Giant Panda [92], Mountain Bongo [93], North goat [94], and Takin [95], are presently underway. The 2006 IUCN Red List of Threatened Species (www.iucnredlist.org) identifies 11 cervid species as either vulnerable or endangered worldwide. Since cervid species frequently hybridize and give rise to fertile offspring [96], there is the opportunity of using red deer as cytoplast donors for NT and as surrogate mothers for ET. This interspecies approach has been successful before, using domestic sheep and cats for cloning closely related wild mouflon sheep and wildcats, respectively [9, 15]. The relatively high success rate of red deer cloning provides encouragement that the technology will be either indirectly (i.e., by using red deer as egg donors for NT and as surrogate mothers for ET) or directly applicable to other cervid species, such as the critically endangered Père David's deer (*Elaphurus davidianus*).

In conclusion, our experiments show that red deer can be cloned with standard NT techniques; however, consistent with previous results, using adult somatic stem cells as donors did not result in obvious improvements of cloning efficiency [97, 98]. We have also established mature adipocytes as a new donor cell type that is easily produced in vitro, is morphologically and molecularly well defined, and results in high electrical fusion and relatively high cloning efficiency.

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