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Myogenic Differentiation of Dermal Papilla Cells From Bovine Skin

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Cells from the dermal papilla and dermal sheath of hair follicles exhibit pronounced plasticity in vitro, being capable of adopting fat, bone, hematopoietic, and nerve cell phenotypes. In this study, we show that bovine dermal papilla cells (DPC) are also capable of undergoing skeletal muscle differentiation. *DiI* labeled DPC incorporated into myotubes when co-cultured with differentiating C_2C_{12} myoblasts. Bovine-specific PCR assays showed that the muscle markers MyoD and myogenin were up-regulated, confirming that the DPC had adopted a myogenic gene expression program. Nine clonal lines of DPC underwent both adipogenic and myogenic differentiation, demonstrating the multipotency of individual cells. Primary populations of both DPC and extra-follicular dermal fibroblasts were also capable of both adipogenic and myogenic differentiation. However, on myogenic differentiation, cells derived from dermal papillae expressed higher levels of myogenin than primary fibroblasts derived from extra-follicular dermis, suggesting that papilla cells undergo myogenesis more efficiently. This result shows that populations of fibroblastic cells derived from different anatomical sites within the skin are not equivalent with respect to their plasticity. Cultured DPC and dermal fibroblasts both expressed Pax3, a marker for the dermomyotome which represents a common embryological origin of muscle and dermis. Quantitative PCR showed that Pax3 expression levels before myogenic induction correlated with myogenin expression levels after myogenesis. These results suggest that a degree of dedifferentiation may underlie the plasticity of dermal cells in vitro, and that this plasticity may be predicted, at least in part, by levels of Pax3 expression. J. Cell. Physiol. 209: 959–966, 2006. © 2006 Wiley-Liss, Inc.

Cells from the dermal compartments of hair follicles, the dermal papilla (DP) and dermal sheath (DS), are thought to participate in wound healing by repopulating the inter-follicular dermis (Jahoda and Reynolds, 2001). Implanted DS cells have been shown to incorporate into healing wounds (Gharzi et al., 2003). In addition to their stem cell-like behavior, these cells exhibit pronounced morphogenetic properties. In appropriate circumstances, they can induce regeneration of amputated follicles or de novo follicle development in adult skin (Jahoda et al., 2001; McElwee et al., 2003). During follicle growth cycling, there is evidence for exchange of cells between DP and DS compartments (Tobin et al., 2003). More extensive plasticity of these cells has been indicated in a report that they can adopt blood cell phenotypes in vitro and repopulate the hematopoietic system of gamma-irradiated mice (Lako et al., 2002). Clonally derived lines of DP and DS cells undergo both adipogenic and osteogenic differentiation in vitro (Jahoda et al., 2003; Richardson et al., 2005a), demonstrating the multipotency of individual cells. Clonally derived lines of cells isolated from the entire dermis, termed skin-derived precursors (SKP), are capable of differentiating into neurons, glial cells, adipocytes, and smooth muscle cells (Toma et al., 2001; Joannides et al., 2004). SKPs can be derived from isolated DP and continue to express DP markers in vitro, suggesting they also represent a multipotent population of follicular dermis cells (Fernandes et al., 2004; Richardson et al., 2005b).

Fibroblasts derived from skin have been shown to undergo myogenic differentiation both in vivo (Pye and Watt, 2001; Montanaro et al., 2003) and in vitro (Salvatori et al., 1995; Wise et al., 1996). However, the dermis contains several sub-populations of fibroblastic cells, each located in distinct anatomical sites and possessing distinct molecular phenotypes (Sorrell and Caplan, 2004). Hair follicle-associated arrector pili muscles also reside within the dermis and could contribute cells to primary cultures (Poblet et al., 2004). To date, the anatomical origin of the dermal cells that possess myogenic potential has not been determined.

Existing data suggest that fibroblasts derived from whole skin may vary in their myogenic potential. Such cells have been reported to engraft damaged muscle tissue in vivo, incorporating into regenerating myofibers (Pye and Watt, 2001; Montanaro et al., 2003). Other authors have reported that dermal fibroblasts fail to engraft myofibers in vivo unless forced to express MyoD, and have suggested the discrepancy results from the use of different lines of fibroblasts (Huard et al., 1998). In vitro, dermal fibroblasts can up-regulate muscle-specific marker genes and incorporate into myotubes when co-cultured with myoblasts, conditioned medium from myoblasts, or medium containing the lectin, Galectin-1 (Salvatori et al., 1995; Wise et al., 1996; Goldring et al., 2000, 2002). The proportion of fibroblasts converting to a myogenic phenotype increases with increasing concentration of conditioned medium, or with increasing duration of treatment (Goldring et al., 2000, 2002). Clonal lines of fibroblasts maintained in these media show greater myogenic conversion frequencies than uncloned, whole population cultures. Thus it appears that only a sub-population of dermal fibroblasts is capable of adopting a myogenic phenotype, and that this sub-population can be selected under appropriate culture conditions.

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Both skeletal muscle and dorsal dermis derive from the embryonic dermomyotome (Christ and Brand-Saberi, 2002; Olivera-Martinez et al., 2004; Ben-Yair and Kalcheim, 2005). Cells leave the dermomyotome, moving beneath the ectoderm to form the dorsal dermis, or to sites of nascent muscle formation where they undergo myogenic differentiation. These shared embryonic origins raise the question of whether dermal cell conversion to a muscle phenotype involves an intermediate dedifferentiation step in which the dermal cells revert to a more dermomyotome-like phenotype. The transcription factor Pax3 is a marker of dermomyotomal development (Epstein, 2000; Christ and Brand-Saberi, 2002; Buckingham et al., 2003). Pax3-expressing dermomyotomal cells appear to be multipotent (Christ and Brand-Saberi, 2002; Ben-Yair and Kalcheim, 2005). Pax3 expression is maintained in muscle progenitor cells as they migrate to sites of muscle formation, and persists in the satellite cells (progenitors) of at least some adult muscles (Conboy and Rando, 2002; Buckingham et al., 2003; Relaix et al., 2005). However, Pax3 is down-regulated as cells undergo myogenic differentiation. Similarly in skin, Pax3 is down-regulated in the developing dermis (Ben-Yair and Kalcheim, 2005). It cannot be detected in adult dermal tissues in vivo, by either immunohistochemistry (Lang et al., 2005) or microarray analysis (Rendl et al., 2005). Thus Pax3 expression seems to correlate with an undifferentiated state in cells of dermomyotomal origin.

In this study, we show that cells derived from bovine DP are capable of undergoing both adipose and myogenic differentiation. We provide evidence that cells from the DP (DPC) undergo myogenic differentiation more efficiently than fibroblasts from extra-follicular dermis (DF). Furthermore, we show that Pax3 is reexpressed by adult dermal cells in vitro, and that expression levels correlate with the efficiency of myogenic differentiation.

METHODS

Tissue dissection and isolation

Skin was collected from the rump or neck of heifers at a local abattoir, within 1 h of death, and kept on ice in dermal cell medium (Table 1). Subsequent dissection steps were carried out at room temperature in a sterile cabinet, using watchmakers' forceps, microscissors, scalpels, 26½ G hypodermic needles and a *SZX9* stereomicroscope (Olympus, Tokyo, Japan). Connective tissue and lower dermis were cleared away from the inner surface of skin samples to expose the lowermost end-bulbs of hair follicles. Follicles were then excised, cleaned of remaining connective tissue and placed in separate drops of medium. A needle was used to remove the DP and clear away the surrounding tissue. Isolated DP were transferred to a culture vessel containing cloning medium to initiate cell growth.

To isolate extra-follicular dermis, the lowermost end-bulbs of hair follicles were exposed in only a portion of the sample. Microscissors were used to remove small pieces of dermis from regions where the follicles were not exposed, at a level just below the bases of the lowermost follicles. Six explants of dermis were placed in each of two 35 mm dishes and air-dried briefly to aid attachment before cloning medium was added to initiate cell growth.

Initiation and maintenance of cell cultures

Dermal papilla cells (DPC) clones were isolated from three animals, as previously described for rat vibrissae (Jahoda et al., 2003). Single DP was placed in wells of 4-well miniplates containing 1 ml medium. After 7 days at 37°C/5% CO_2, outgrowing cells surrounding each explant were counted, harvested by trypsinization, and resuspended in cloning medium at approximately 1 cell/50 μl. Aliquots (25, 50, or 100 μl) were added to wells of a 96-well plate, made to 200 μl/well final volume, and maintained at 37°C/5% CO_2, with weekly medium changes. After 10–16 days, clones were passaged into 6-well plates or 35 mm dishes. After a further 6–14 days, confluent cells were passaged again into 25 cm^2 flasks. Cells were progressively changed from cloning medium to dermal cell medium during the first and second passages. Cells were subsequently maintained in 75 cm^2 flasks or 10 cm dishes. Aliquots of cells were frozen in liquid nitrogen at passage 2 or 3. Duplicate primary cultures of DPC and DF were initiated as described above. Outgrowth of cells was seen after 7 days, from which time the medium was changed twice weekly. Cells were passaged into 25 cm^2 flasks after 14 days and were subsequently maintained in dermal cell medium. Confluent cells were passaged again into 75 cm^2 flasks (after 3 days for DF, 10 days for DPC). Aliquots of passage 2 cells were frozen in liquid nitrogen. For adipose and myogenic differentiation experiments, single aliquots of dermal cells were thawed, seeded into 10 cm dishes in dermal cell medium, grown to confluence and harvested by trypsinization.

Phase contrast images were photographed on an *Axiovert 40CFL* microscope (Zeiss, Göttingen, Germany) with a *DC330E* digital camera (MTI, Michigan City, IN). Fluorescent and bright-field images were photographed on a *BX50* microscope (Olympus) with a *SPOT* digital camera (Diagnostic Instruments, Sterling Heights, MI).

Adipose differentiation and staining

DPC or DF in dermal cell medium were seeded into *SonicSeal* slides (Nunc, Roskilde, Denmark) at a density of 76,000 cells/well (1.54 cm^2). Cells were changed to adipogenic medium (Jahoda et al., 2003) the day after seeding, with further medium changes twice weekly. Negative controls were maintained in dermal cell medium. After 7–43 days, cells were stained for lipid accumulation: cells were washed briefly in PBS, fixed in 4% formaldehyde/1% calcium chloride for 1 h, incubated in 60% isopropanol for 15 min, stained in *Oil Red O* (Sigma St. Louis, MO, three parts saturated solution in isopropanol: two parts water) for 15 min, washed briefly in 60% isopropanol, and then washed thoroughly in distilled water.

Co-culture with C_2C_{12} cells and staining

DPC or DF in dermal cell medium were harvested by trypsinization, counted, pelleted and resuspended in MEM (no additives) at 10^6 cells/ml. *DiI* stock solution (Molecular

TABLE 1. Tissue culture media

Medium	Composition[a]
Dermal cell medium	MEM, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 12.5 ng/ml *fungizone*, 10% fetal calf serum
C_2C_{12} medium	DMEM, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 12.5 ng/ml *fungizone*, 10% fetal calf serum
Adipogenic medium	MEM, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 12.5 ng/ml *fungizone*, 15% rabbit serum, 0.45 nM isobutyl-methylxanthine, 2.07 μM insulin, 100 nM dexamethasone
Myogenic medium	DMEM, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 12.5 ng/ml *fungizone*, 2% horse serum
Cloning medium	Dermal cell growth medium supplemented with 20% DPC-conditioned medium
DPC-conditioned medium	MEM, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 12.5 ng/ml *fungizone* exposed to primary DPC for 20 h at 37°C/5% CO_2

[a]Media and reagents were purchased from Gibco-Invitrogen (Carlsbad, CA) and Sigma (St. Louis, MO).

Probes, Eugene, OR) was added at 5 μl per 1,000 μl of cells, at 37°C for 20 min. Stained cells were pelleted and resuspended in dermal cell medium at 10^6 cells/ml, three consecutive times, to wash away unbound *DiI*. Cells were recounted before the final resuspension and diluted to 40,000 cells/ml. C_2C_{12} myoblasts were a gift from Mark Thomas (AgResearch) and were maintained in C_2C_{12} medium (Blau et al., 1983). Passage 13–30 C_2C_{12} cells were harvested by trypsinization and resuspended in C_2C_{12} medium at 40,000 cells/ml. DPC or DF were mixed with C_2C_{12} cells before seeding into *SonicSeal* slides at a combined density of 76,000 cells/well. Cells were seeded in ratios of 0:100%, 20:80%, 50:50%, 80:20%, and 100:0% dermal cells: C_2C_{12} cells. All cells were changed to myogenic medium the day after seeding, with further medium changes twice weekly. After 4–10 days, cells were counter-stained with 5 μg/ml *Hoechst 33342* stain (Sigma) in DMEM at 37°C for 10 min, washed three times for 3 min in myogenic medium at 37°C, fixed in 10% buffered formalin for 5 min, washed three times for 3 min in PBS, and mounted using *AquaMount* (BDH, Poole, UK). Cells were kept in the dark as much as possible throughout.

RNA extraction, reverse transcription, and PCR

For RNA extraction and PCR analysis, co-cultures were set up and maintained as described above, except that DPC and DF were not labeled with *DiI*. After 4–10 days in myogenic medium, RNA was extracted using a *Micro-to-midi* kit (Invitrogen, Carlsbad, CA) according to the supplied protocol, eluted in 30 μl RNase-free water and treated with DNase I using a *DNA-free* kit (Ambion, Austin, TX). cDNA was synthesized from 150 to 500 ng RNA using random hexamer primers and a *Superscript III* kit (Invitrogen) according to the supplied protocol. Bovine-specific PCR primers (Sigma-GenoSys, Sydney, Australia) were designed using Primer3 software (Rozen and Skaletsky, 2000) and targeted sites not conserved in the mouse orthologue of the relevant gene (Table 2).

End-point PCRs were performed in 15 μl reactions, incorporating 1 μl cDNA template (reagents from Invitrogen). Template loadings were normalized by diluting cDNA in TE to achieve equal amplification of GAPDH, using the Bt-Mm-GAPDH-for/-rev primers (Table 2). Amplification conditions were as follows: GAPDH, 1.5 mM $MgCl_2$, 55°C annealing temperature, 30 cycles; myogenin, 1.0 mM $MgCl_2$, 53°C annealing temperature, 38 cycles; MyoD, 0.7 mM $MgCl_2$, 54°C annealing temperature, 40 cycles. Aliquots (7.5 μl) of the completed reactions were subjected to electrophoresis in 1.5–2.0% agarose/TAE gels stained with *SybrSafe* (Molecular Probes) and photographed under UV illumination.

Quantitative PCR was performed using a *LightCycler* real-time PCR instrument and software (Roche, Mannheim, Germany) with a *LightCycler FastStart DNA Master SYBR Green* kit (Roche), according to the manufacturers instructions. Standard curves were prepared from twofold dilution series of cDNA synthesized with 1 μg selected RNA samples. Bovine-specific primers for myogenin, Pax3, and GAPDH (Table 2) were incorporated in 9 μl of PCR master mixture added to 1 μl of cDNA. PCR conditions were as follows: 10 min activation at 95°C; 35 (GAPDH) or 42 (Myogenin and Pax3) cycles of amplification at 95°C for 10 sec, 60°C for 10 sec, and 72°C for 10 sec. Melting curve analysis confirmed amplification of a single product in each assay. Expression values for myogenin and Pax3 were normalized to bovine GAPDH.

RESULTS

Dermal papilla cell clones

Three clonal lines of DPC were isolated from each of three animals. Clones varied in cell morphology (Fig. 1A–D), but all expressed the DPC marker alpha-actin as determined by quantitative PCR (not shown). All nine clones underwent similar adipose differentiation when maintained in adipogenic medium (Fig. 1E–H). By 7 days, most cells stained positively for lipid, as determined by *Oil Red O* staining. No *Oil Red O* staining was seen in cells maintained in dermal cell medium. No differences between clones were observed in the extent of adipose differentiation.

The myogenic potential of DPC clones was explored by determining whether they could incorporate into myotubes formed by differentiating C_2C_{12} cells. DPC were labeled with *DiI*, mixed with unlabeled C_2C_{12} cells and maintained in myogenic medium. As *DiI* labels the membrane of cells and can diffuse throughout this compartment, the presence of multinucleated myotubes labeled with *DiI* would indicate the incorporation of DPC. In practice, *DiI* labeled myotubes were seen from 4 to 10 days after co-cultures were placed in myogenic medium (Fig. 1I–P). All nine DPC clones incorporated into differentiating myotubes, with no differences between DPC clones in the abundance of labeled myotubes or the timing of differentiation. DPC clones and C_2C_{12} cells were co-cultured at ratios of 20:80%, 50:50%, and 80:20%, but the cell ratio had no effect on myotube formation. No fluorescent myotubes were seen when C_2C_{12} cells differentiated in the absence of DPC, excluding the possibility of myotube autofluorescence. No fluorescent myotubes were seen when DPC were maintained in myogenic medium in the absence of C_2C_{12} cells. Thus we found no evidence of spontaneous myogenic differentiation of DPC clones.

In order to demonstrate full myogenic conversion of DPC, it was necessary to demonstrate that their nuclei had been reprogrammed to express muscle-specific genes, and to exclude the possibility that the DPC were fusing with myotubes passively and not changing their nuclear phenotype. We addressed this issue by establishing RT-PCR assays that distinguish between DPC-derived bovine homologues and C_2C_{12}-derived murine homologues of two muscle marker genes, MyoD and myogenin. These transcription factors are involved in muscle determination and differentiation, respectively (Christ and Brand-Saberi, 2002; Buckingham et al., 2003). The bovine-specificity of the assays was confirmed by demonstrating a negative result for C_2C_{12} cells differentiating in the absence of DPC (Fig. 2C), and by sequencing the bands shown in Figure 2A,B.

TABLE 2. PCR primers

Primer	Sequence	Specificity	GenBank ACC numbers
Bov-myogenin-for	GGTCTGGGGCCTGCG	Bovine only	Bovine, AB110600
Bov-myogenin-rev	GTAGGCACTCTCGGG	Bovine only	Murine, NM_031189
Bov-MyoD-for	GCCGGGAGCGAGGTG	Bovine only	Bovine, AB110599
Bov-MyoD-rev	AGTCCCAGGGAGCGC	Bovine only	Murine, NM_010866
Bt-Pax3-for-2	ATGTTCAGCTGGGAAATTC	Bovine only	Bovine, XM_613826
Bt-Pax3-rev-2	TCTGAACGGGGACTTCTC	Bovine only	Murine, NM_008781
Bov-GAPDH-for	TCACCAGGGCTGCTTTTAAT	Bovine only	Bovine, U85042
Bov-GAPDH-rev	AGGACGGTTGTAGTTCACC	Bovine only	Murine, M32599
Bt-Mm-GAPDH-for	TTCAACGGCACAGTCAAGG	Bovine and murine	Bovine, U85042
Bt-Mm-GAPDH-rev	CTACGGGGGTACAAACACT	Bovine and murine	Murine, M32599

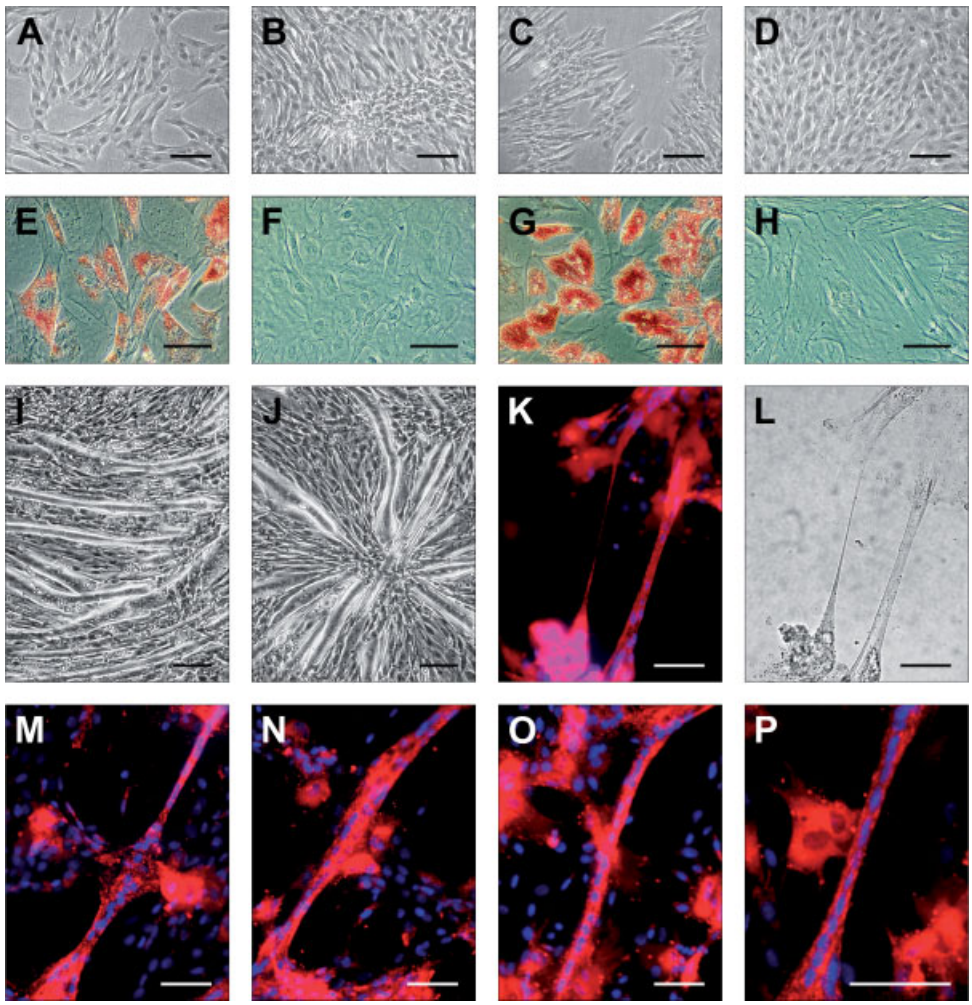


Fig. 1. Adipogenic and myogenic differentiation of bovine dermal papilla cell clones. **A**–**D**: Clonal lines showed different cell morphologies before differentiation. Clones 1-A4 (A), 1-A7 (B), 1-F11 (C), and 1-H7 (D) were derived from the same animal. **E**–**H**: Adipose differentiation of clones 1-A4 (E, F) and 1-E5 (G, H). Cells were maintained in adipogenic medium (E, G) or dermal cell medium (F, H) for 25 days and then stained with *Oil Red O*. **I**–**P**: Myogenic differentiation of clones co-cultured with C_2C_{12} cells. Clones tended to promote the formation of myotubes in either parallel (I, clone 1-C3) or radial patterns (J, clone 1-F11). *DiI*-labeled DPC (red) were co-cultured with unlabeled C_2C_{12} cells and counterstained with *Hoechst 33342* (blue nuclei). Shown are 50% co-cultures of (K, L) clone 2-F1 after 6 days, (M) clone 1-C3 after 6 days, (N) clone 3-E3 after 6 days, (O) clone 1-C3 after 6 days, (P) clone 2-FI after 5 days. (L) bright-field image of the field shown in (K). Scale bars, 100 μm.

Using these assays, expression of bovine MyoD and myogenin mRNA was determined in co-cultures of unlabeled DPC with C_2C_{12} cells (Fig. 2A,B). Bovine MyoD and myogenin expression were detected in co-cultures of all nine DPC clones, indicating that all nine were indeed adopting a myogenic gene expression program. DPC were co-cultured with C_2C_{12} cells at ratios of 20:80%, 50:50%, and 80:20%, and expression was determined at three time-points from 4 to 10 days after addition of myogenic medium. No difference in myogenin expression was seen between clones, time-points or co-culture ratios. There was some variation in MyoD band intensities, apparently random, but expression was detected for all clones, at all time-points and co-culture ratios. Bovine MyoD and myogenin expression were not detected for any clone at any time-point when DPC were maintained in the absence of C_2C_{12} cells (Fig. 2A,B). Thus the DPC clones did not show spontaneous expression of these muscle markers.

Primary dermal cells

Dermal cells derived from distinct anatomical sites may vary in their differentiation potential. Possible diversity among dermal cells from bovine skin was explored by determining the adipogenic and myogenic potential of uncloned primary cells, derived from both dermal papillae and from small explants of extra-follicular dermis (Fig. 3A). Primary cells were chosen to be more representative of their tissue of origin, as non-representative sub-populations might be selected during the cloning process. Separate populations of primary cells were grown from two batches of papillae and two batches of extra-follicular dermis. All four populations of cells had a fibroblastic appearance, but cells derived from extra-follicular dermis (DF) had a more "spindly" morphology with more pronounced processes extending from the cell body (Fig. 3B,C). When confluent, DPC appeared more closely-packed than DF (Fig. 3D,E). All four populations underwent adipose differentiation when maintained in adipogenic medium (Fig. 3F,G). *Oil Red O* staining showed lipid droplet accumulation in the cytoplasm of most cells by 8 days. No *Oil Red O* staining was seen in cells maintained in dermal cell medium. There were no differences between the four populations of cells in the extent of adipose differentiation.

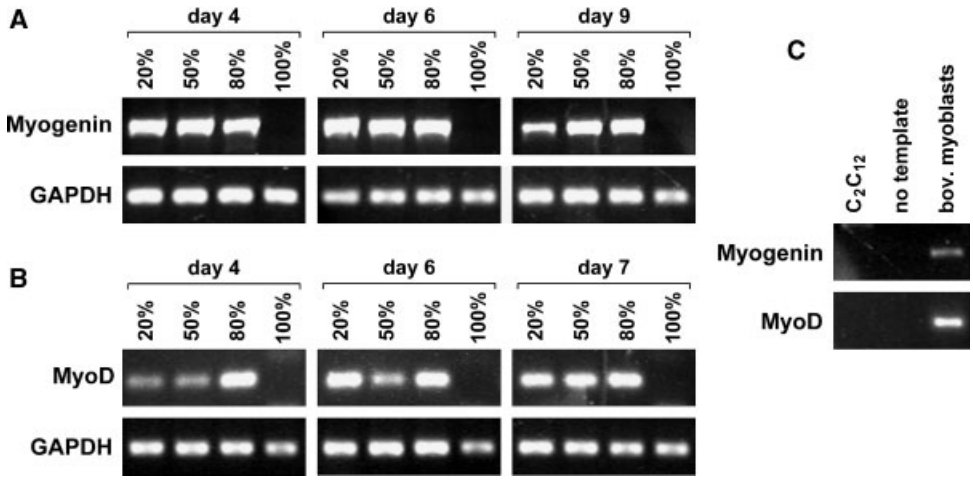


Fig. 2. End-point PCR analysis of MyoD and myogenin expression in co-cultures of dermal papilla cell clones and C_2C_{12} cells. **A**, **B**: Clones and C_2C_{12} cells were co-cultured in ratios of 20:80%, 50:50%, 80:20%, or 100:0% in myogenic medium. Percentages of dermal cells in cultures are shown. GAPDH primers amplified both bovine and murine targets, hence bands represent the total number of cells of both species. A: Myogenin expression in co-cultures of clone 3-E3 at 4 (t = 1), 6 (t = 2), and 9 days (t = 3) exposure to myogenic medium. B: MyoD expression in co-cultures of clone 2-C6 at 4 (t = 1), 6 (t = 2), and 7 days (t = 3) exposure to myogenic medium. **C**: Controls confirming bovine-specific amplification of myogenin and MyoD.

To compare the myogenic potential of primary dermal cells, they were labeled with *DiI*, mixed with unlabeled C_2C_{12} cells in a 50:50% ratio, and placed in myogenic medium. Labeled myotubes were seen after 4–10 days, for all four populations of cells (Fig. 3H–K). Bovine-specific PCR assays were used to determine myogenin and MyoD expression in co-cultures of unlabeled primary cells with C_2C_{12} cells. Bovine myogenin expression was detected in all four populations, at all three time-points after addition of myogenic medium (Fig. 3L,M). Expression was not seen in primary cells maintained without C_2C_{12} cells, except for the DP-2 population after 8 days. This may represent an example of spontaneous muscle differentiation, as has been reported for follicular dermal cells of other species (Jahoda et al., 2003).

We could not reproducibly detect bovine MyoD expression in co-cultures of primary dermal cells and C_2C_{12} cells. This contrasts with the results seen for DPC clones cultured and assayed under the same conditions (Fig. 2B). It may indicate that primary cells undergo muscle differentiation with lower efficiency than the clones, such that the levels of bovine MyoD mRNA in the co-cultures are below the sensitivity of the assay.

Pax3 expression

Pax3 expression levels were determined to explore the possibility that myogenic differentiation of dermal cells involves dedifferentiation to a more dermomyotomal-like state. Pax3 expression could be detected by end-point PCR in cultured dermal cells (not shown). This contrasts with its reported lack of expression in dermal tissues in vivo (Ben-Yair and Kalcheim, 2005; Lang et al., 2005; Rendl et al., 2005), suggesting Pax3 is up-regulated when dermal cells are introduced to culture.

We then used real-time PCR to quantify Pax3 expression in the nine DPC clones and four primary populations before myogenesis, that is, in the absence of C_2C_{12} cells. We similarly quantified myogenin expression after co-culture with C_2C_{12} cells to induce myogenic differentiation. The results are summarized in Figure 4. The primary DF appear distinct from DP derived cells, with lower expression of both Pax3 and myogenin. This indicates that the DF cells represent a distinct phenotype which undergoes myogenic differentiation less efficiently than DPC. The primary DPC also showed significantly lower levels of myogenin expression than the DPC clones. These results are consistent with our inability to detect MyoD expression in co-cultured primary cells, further indicating that they undergo myogenic differentiation less efficiently than the DPC clones.

With the exception of two outliers, there was a positive correlation between expression of Pax3 before myogenesis and myogenin expression afterwards. Inspection of the raw data suggested that the high myogenin outlier (Fig. 4A, upper left) arose from an anomalously low GAPDH value, rather than high myogenin. The high Pax3 outlier (Fig. 4A, lower right) represents the same primary population that showed evidence of spontaneous myogenic differentiation in the absence of C_2C_{12} cells (Fig. 3L). In comparing this DPC population at three consecutive time-points, the first exhibited high Pax3 expression, the second showed similar Pax3 expression to other DPC, and only the third showed spontaneous myogenin expression. This time-course is reminiscent of the down-regulation of Pax3 seen prior to myogenic differentiation in vivo (Epstein, 2000; Christ and Brand-Saberi, 2002; Buckingham et al., 2003; Relaix et al., 2005). Overall, the correlation between expression of the two genes suggests that Pax3 levels predict the susceptibility of dermal cells to myogenic differentiation when placed in an inductive environment.

DISCUSSION

We have shown that DPC are capable of adopting a myogenic fate when co-cultured with C_2C_{12} cells, incorporating into myotubes and expressing muscle commitment and differentiation markers. All nine of the DPC clones that we studied were also capable of adipose differentiation, demonstrating the multipotency of individual cells. Our adipose differentiation results are equivalent to those previously reported for cells from rat vibrissae (Jahoda et al., 2003) and human follicles (Richardson et al., 2005a), except that only a subset of rat clones were capable of adipogenic differentiation. It is not clear whether differences in the consistency of

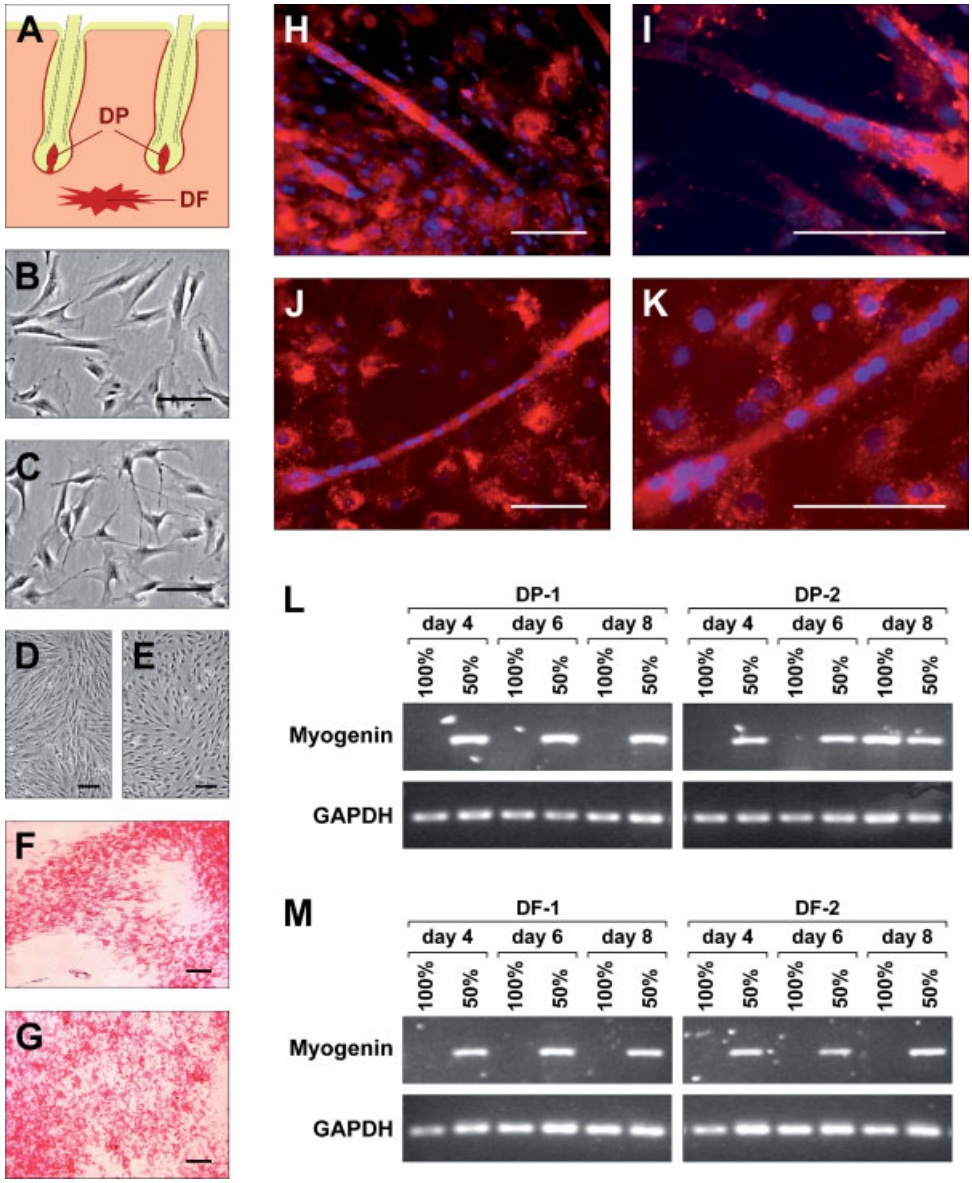
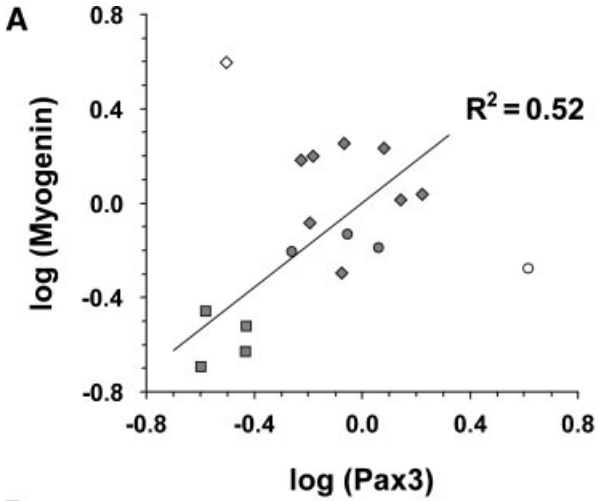


Fig. 3. Adipogenic and myogenic differentiation of primary populations of bovine dermal papilla cells and extra-follicular dermal fibroblasts. **A**: Diagram showing sources of tissue for establishing primary DPC and DF cultures. **B**–**E**: Morphology of primary cells. B: Sub-confluent DPC, (C) sub-confluent DF, (D) confluent DPC, and (E) confluent DF. **F**, **G**: Adipose differentiation of DPC (F) and DF (G). Cells were maintained in adipogenic medium for 8 days and then stained with *Oil Red O*. **H**–**K**: Myogenic differentiation of primary DPC and DF populations co-cultured with C_2C_{12} cells. *DiI*-labeled dermal cells (red) were co-cultured with unlabeled C_2C_{12} cells at a ratio of 50:50% and counterstained with *Hoechst 33342* (blue nuclei). H: DP-2 after 6 days in myogenic medium, (I) DP-1 after 5 days, (J) DF-1 after 7 days, and (K) DF-2 after 8 days. Scale bars, 100 μm. (**L**, **M**) End-point PCR analysis of myogenin expression in co-cultures of two DPC populations (L) and two DF populations (M). Dermal cells were mixed with C_2C_{12} cells at a ratio of 50:50% or 100:0% and maintained in myogenic medium for 4 (t = 1), 6 (t = 2), or 8 (t = 3) days. Percentages of dermal cells in cultures are shown. RT-PCR was performed using bovine-specific primers for myogenin. GAPDH primers amplified both bovine and murine targets, hence bands represent the total number of cells of both species.

adipogenic differentiation relate to the species or follicle type (vibrissa versus pelage) from which the cells were isolated.

We found that uncloned primary populations of DPC exhibited similar myogenic and adipogenic activity to DPC clones, suggesting the clones were representative of cells found in primary cultures. Myogenic differentiation involves the fusion of cells to form multinucleated myotubes. Thus, to demonstrate true myogenic conversion of dermal cells, it was necessary to show that the dermal cell nuclei were reprogrammed to express muscle-specific genes. We developed bovine-specific PCR assays to show expression of bovine MyoD and myogenin in chimaeric myotubes, thereby demonstrating that the dermal cell nuclei had adopted a muscle-specific gene expression program. These results broaden the known plasticity of DPC, adding myogenic differentiation to a range of phenotypes that can be induced under appropriate conditions (Lako et al., 2002; Jahoda et al., 2003; Fernandes et al., 2004; Richardson et al., 2005a).

Other workers have found that fibroblastic cells derived from skin are similarly capable of myogenic differentiation (Salvatori et al., 1995; Wise et al., 1996). However, these cells were isolated from skin without attempting to separate discrete compartments of dermal



B

	t-test probabilities	
	log (Pax3)	log (Myogenin)
1° DF *vs* 1° DP	0.028	0.002
1° DF *vs* DP clones	0.0001	0.00002
1° DP *vs* DP clones	0.686	0.008

Fig. 4. Quantitative PCR analysis of Pax3 and myogenin expression. Pax3 expression was measured in 100% dermal cells maintained in myogenic medium. Pax3 expression was not detected in C_2C_{12} cells, confirming the assay was bovine-specific. Myogenin expression was measured in 50:50% dermal cells: C_2C_{12} cells maintained in myogenic medium. Expression of both genes was normalized to GAPDH expression using bovine-specific primers. **A**: The log of normalized myogenin expression is plotted against the log of normalized Pax3 expression for nine DPC clones at t = 1 (diamonds), two primary DPC populations at t = 1 and t = 2 (circles), and two primary DF populations at t = 1 and t = 2 (squares). Two outliers are shown with unshaded symbols. The regression line and co-efficient were calculated excluding the outliers. **B**: Summary of *t*-tests for differences in expression of Pax3 and myogenin between DPC clones, primary DPC, and primary DF. Two-tailed tests were performed assuming unequal variance. The outliers shown in (A) were excluded.

tissues. Considering that skin contains several distinct mesenchymal tissues as well as the arrector pili muscles associated with hair follicles, it is important to determine which cells in skin have myogenic potential. We grew cells from explants that were carefully microdissected from defined tissue compartments, ensuring the origin of the cells was known. All DPC clones expressed alpha-actin, an established marker of follicular dermal cells (Reynolds et al., 1993), and all exhibited myogenic potential. The DF we isolated were morphologically distinct from the DPC. Primary cultures of DF as well as DPC exhibited both myogenic and adipogenic activity. However, the DF expressed lower levels of myogenin, suggesting they undergo myogenic differentiation less efficiently. It has been proposed that dermal cells from hair follicles repopulate the surrounding dermis during wound healing (Jahoda and Reynolds, 2001; Gharzi et al., 2003). Our results are consistent with the possibility that extra-follicular dermal cells represent a derived, more differentiated, and less plastic state than DPC.

The adoption of a myogenic phenotype by dermal cells is interesting in light of the common developmental origins of skeletal muscle and dorsal dermis (Christ and Brand-Saberi, 2002; Olivera-Martinez et al., 2004; Ben-Yair and Kalcheim, 2005). Both are derived from the dermomyotome, raising the possibility that myogenic differentiation of dermal cells involves a dedifferentiation step in which they revert to a more dermomyotome-like phenotype. The transcription factor Pax3 is a marker for the dermomyotome, but is down-regulated in differentiated muscle and dermal tissues (Epstein, 2000; Christ and Brand-Saberi, 2002; Conboy and Rando, 2002; Buckingham et al., 2003; Ben-Yair and Kalcheim, 2005). Our results suggest that Pax3 is reexpressed when bovine dermal cells are introduced to culture. Similarly, murine SKPs have been shown to express Pax3 (Fernandes et al., 2004). Thus, with respect to this gene at least, cultured dermal cells appear closer to a dermomyotomal phenotype than their counterparts in vivo. We found that Pax3 expression levels before myogenesis were correlated with the efficiency of myogenin up-regulation on co-culture with C_2C_{12} cells. This relationship may be causal. Pax3-expressing dermomyotomal cells migrate to both nascent muscle and dermis, suggesting they are multipotent (Christ and Brand-Saberi, 2002; Ben-Yair and Kalcheim, 2005). Mutant mouse phenotypes indicate that Pax3 is necessary for myogenic fate specification in vivo (Tajbakhsh et al., 1997; Epstein, 2000). However, in C_2C_{12} cells, forced Pax3 expression inhibits myogenic differentiation (Epstein et al., 1995). Thus Pax3 functions to facilitate cell fate determination processes while preventing terminal differentiation. Up-regulation of Pax3 may be a prerequisite for dermal cells to enter a more plastic state from which they can then adopt other phenotypes.

Pax3 is also expressed in cells of the neural crest lineage, where it seems to have a similar function. In Schwann cells, Pax3 expression inhibits myelin basic protein expression and terminal differentiation (Kioussi et al., 1995). In melanocytes, Pax3 stimulates expression of the determination factor, Mitf, while simultaneously inhibiting terminal differentiation (Lang et al., 2005). The B16/F10.9 melanoma cell line can be induced to transdifferentiate to a Schwann cell phenotype by treatment with a recombinant IL6 protein (Slutsky et al., 2003). Transdifferentiation is associated with down-regulation of Pax3, and is inhibited by forced expression of Pax3. Thus as for dermal cells, Pax3 expression in neural crest-derived cells may confer a more plastic state, facilitating fate determination while preventing terminal differentiation.

In summary, we have shown that bovine DPC can undergo myogenic differentiation in vitro. They do so more efficiently than cells from the extra-follicular dermis. This difference correlates with differences in expression of Pax3, which may be significant for the plasticity of these cells. If these results extend to humans, DPC may be a better choice than DF for therapeutic applications involving multipotent cells from skin.

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