Human skeletal muscle fibroblasts, but not myogenic cells, readily undergo adipogenic differentiation

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Summary

We characterised the adherent cell types isolated from human skeletal muscle by enzymatic digestion, and demonstrated that even at 72 hours after isolation these cultures consisted predominantly of myogenic cells (CD56$^+$, desmin$^+$) and fibroblasts (TE-7$^+$, collagen VI$^+$, PDGFRα^+, vimentin$^+$, fibronectin$^+$). To evaluate the behaviour of the cell types obtained, we optimised a double immuno-magnetic cell-sorting method for the separation of myogenic cells from fibroblasts. This procedure gave purities of >96% for myogenic (CD56$^+$, desmin$^+$) cells. The CD56$^-$ fraction obtained from the first sort was highly enriched in TE-7$^+$ fibroblasts. Using quantitative analysis of immunofluorescent staining for lipid content, lineage markers and transcription factors, we tested if the purified cell populations could differentiate into adipocytes in response to treatment with either fatty acids or adipocyte-inducing medium. Both treatments caused the fibroblasts to differentiate into adipocytes, as shown by loss of intracellular TE-7, upregulation of the adipogenic transcription factors PPARγ and C/EBPα, and adoption of a lipid-laden adipocyte morphology. By contrast, myogenic cells did not undergo adipogenesis and showed differential regulation of PPARγ and C/EBPα in response to these adipogenic treatments. Our results show that human skeletal muscle fibroblasts are at least bipotent progenitors that can remain as extracellular-matrix-producing cells or differentiate into adipocytes.

Key words: Fibroblasts, Adipogenesis, Stem cells, Skeletal muscle, Satellite cells, Transdifferentiation

Introduction

The essential role of satellite cells (Mauro, 1961) in muscle regeneration is well known (Lepper et al., 2011; Sambasivan et al., 2011), but these cells have also been proposed as the source of fat accumulation in mouse (Grimaldi et al., 1997; Asakura et al., 2001; Csete et al., 2001; Wada et al., 2002; Shefer et al., 2004; Vertino et al., 2005; Aguiari et al., 2008; Vettor et al., 2009) and human skeletal muscle (De Coppi et al., 2006). Fat deposition in muscle can be either as intramyocellular lipid droplets (within muscle fibres, which can be either physiological or pathological) or in the form of adipocytes located in the perimysial space (in modest amounts, not uncommon in normal muscle) or within fascicles (replacing muscle fibres) in some pathologies (Schrauwen-Hinderling et al., 2006; Vettor et al., 2009). Indeed, fatty degeneration in skeletal muscle is a hallmark of many myopathies, sarcopenia, obesity and incomplete regeneration (Borkan et al., 1983; Ravussin and Smith, 2002; Tyler, 2003; Wagatsuma, 2007; Delmonico et al., 2009). It has been suggested that satellite cells, which are adult skeletal muscle stem cells (Zammit and Beauchamp, 2001; Zammit et al., 2006; Yin et al., 2013) can be diverted from the myogenic lineage and transformed into adipogenic, fibroblastic, osteogenic and even neuronal cell types under the appropriate culture conditions *in vitro* (Asakura et al., 2001; Wada et al., 2002) and also *in vivo* (Brack et al., 2007; Sarig et al., 2010). However, other recent data from studies of mouse skeletal muscle strongly question the apparent diversion of satellite cells to the adipogenic lineage (Joe et al., 2010; Uezumi et al., 2010; Uezumi et al., 2011). It has been suggested that methodological limitations affecting the isolation of 'pure' satellite cell populations might explain their ostensible changes in fate demonstrated in culture (Starkey et al., 2011).

Recently, a population of undifferentiated 'fibro-adipogenic' precursors (FAPs) isolated from mouse skeletal muscle were reported to differentiate into fibroblasts or adipocytes (Joe et al., 2010; Uezumi et al., 2010). However, the relationship between FAPs and local stromal fibroblasts is unclear (Murphy et al., 2011). Identifying fibroblasts in culture can be problematic, but an increasing body of work has concentrated on identifying specific markers for these cells (Goodpaster et al., 2008; Mathew et al., 2011). TE-7 is a fibroblast-specific connective tissue protein that can be used to identify fibroblasts and distinguish them from human peripheral blood monocyte-derived fibrocytes, endothelial cells, chondrocytes, macrophages and other cell types (Stewart et al., 2003; Pollina et al., 2008; Pilling et al., 2009; Pfisterer et al., 2011). Furthermore, the TE-7 antigen has been carefully validated *in situ* and *in vitro* for the detection of fibroblasts in a number of tissues, including human skeletal muscle (Haynes et al., 1984; Stewart et al., 2003; Goodpaster et al., 2008).

The main aim of our study was to test the potential of myogenic cells and fibroblasts from human muscle to differentiate into adipocytes using two different stimuli; first, a physiologically-relevant nutrient cue, fatty acids (FAs) (Watt et al., 2012) and second, an adipocyte-inducing medium (AIM) (Fritzius and Moelling, 2008; Rajashekhar et al., 2008; Catalioto et al., 2009). To achieve this we optimised a double immuno-magnetic cell-sorting method based on the cell-surface

expression of CD56 on myogenic cells (Cashman et al., 1987; Mackey et al., 2009) for the reproducible purification and subsequent culture of the major cell populations from human muscle. $CD56^{-}$ cells were confirmed as fibroblasts by their TE-7 expression.

Adipogenic differentiation was determined by lipid accumulation, changes in cell morphology, and expression of lineage markers and transcription factors, following exposure to adipogenic stimuli. In addition, we measured the nuclear expression of the master regulators of adipogenesis, peroxisome proliferator activator receptor gamma (PPARγ) and CCAAT/enhancer binding protein alpha (C/EBPα) because these transcription factors are known to trigger the terminal differentiation of preadipocytes into mature adipocytes (Shao and Lazar, 1997; Rosen and Spiegelman, 2006). We also tested our cultures for the presence of cells expressing the CD15 (Lecourt et al., 2010; Pisani et al., 2010) and platelet-derived growth factor receptor alpha (PDGFRα) (Uezumi et al., 2010) cell surface antigens, because these have recently been indicated as markers for two highly adipogenic sub-populations in skeletal muscle.

Our results clearly show that fibroblasts are the most abundant non-myogenic adherent cell population in human skeletal muscle. These cells highly express TE-7 and a battery of other fibroblast lineage markers soon after they attach to the culture dish (72 hours post isolation). Our data indicate that unlike the myogenic cells, which showed considerable resistance to adipogenesis, the fibroblasts have an inherent adipogenic potential, making them the most likely source of adipocytes that accumulate in skeletal muscle in a range of conditions.

Results

In situ localisation of myogenic (CD56) and connective tissue (TE-7) markers

We first demonstrated the *in situ* localisation of CD56 (a myogenic cell surface antigen), TE-7 (a connective-tissue protein) and Oil-Red-O (which stains neutral lipid) on cryosections of human muscle from a young and an elderly person (Fig. 1). Immunostaining for CD56 and laminin revealed satellite cells ($CD56^{+}$) located beneath the basal lamina encasing mature fibres (Fig. 1A,B). The TE-7 antigen was localised in the endomysium

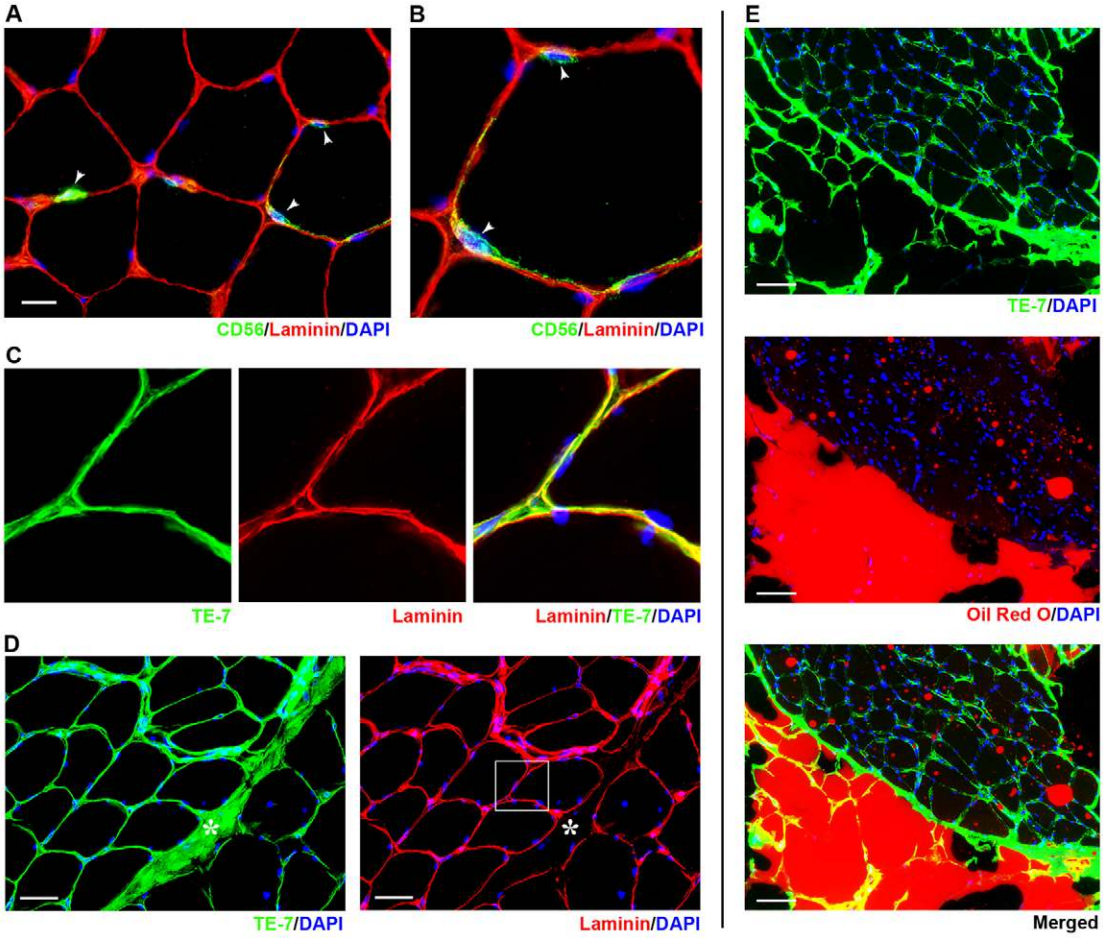

Fig. 1. Immunolocalisation of CD56 and TE-7 on cryosections of human skeletal muscle. (A) Satellite cells are identified by their $CD56^{+}$ status (arrows) and position under the basal lamina (enlarged in B). (C) Comparison of TE-7 and laminin staining indicates that TE-7 is dispersed throughout the endomysium, and merges with the outer surface of the laminin-positive basal lamina. (D) TE-7 reactivity is also present throughout the perimysium (asterisk), which does not have laminin staining; the white box shows the field that is enlarged in C. (E) Typical cryosection of skeletal muscle from an old (age 83) sedentary individual showing a large area of gross intramuscular fat deposition (Oil-Red-O staining) and also strong TE-7 staining around the adipocytes. Scale bars: 20 µm (A); 50 µm (D); 100 µm (E).

surrounding muscle fibres and also partially overlapped anti-laminin staining (Fig. 1C). However, in perimysium, TE-7 was highly expressed, whereas laminin staining was absent (Fig. 1D). Similarly, in muscle from the elderly subject, which included areas of both fibrosis and intramuscular lipid deposition, TE-7 staining was strong in the connective tissue (Fig. 1E). No CD15 staining was detectable on these sections of human muscle.

Early characterisation of human muscle-derived cultures

In order to establish which cell types are present during early stages of culture, human muscle-derived cells were isolated, plated onto collagen-coated coverslips and fixed after 72 hours once they had attached. To quantify the relative abundance of cell types, the expression of lineage markers was evaluated by immunostaining. Fibroblasts were confirmed by their high expression of collagen VI (Zou et al., 2008; Murphy et al., 2011), (supplementary material Fig. S2A,B), TE-7 (supplementary material Fig. S2C), vimentin (Li et al., 2008) (supplementary material Fig. S2D), PDGFRα (Bonner, 2004; Leask, 2010) (supplementary material Fig. S2E) and fibronectin (Kalluri and Zeisberg, 2006) (supplementary material Fig. S3A,C). PDGFRα$^+$ cells were quantified based on an intense punctate staining pattern on the cell membrane. Counts for TE-7 and collagen VI on independent coverslips from the same population were closely matched, confirming the connective tissue lineage and extracellular matrix (ECM)-producing capacity of these cells (supplementary material Fig. S3D). All myogenic cells expressed the surface marker CD56 and the intracellular marker desmin (van der Ven et al., 1992; Boldrin et al., 2010) (supplementary material Fig. S2B–E). Myogenic cells also expressed vimentin (supplementary material Fig. S2D; Fig. S3D) and many expressed fibronectin (supplementary material Fig. S3B). Of the putative fibroblast markers used, TE-7 was the most discriminating for fibroblasts and was completely absent from myogenic cells (supplementary material Fig. S2C; Fig. S3D). At 72 hours post dissociation, fibroblasts and myogenic cells accounted for virtually all of the adherent muscle-derived population (supplementary material Fig. S3D).

Double immuno-magnetic purification of myogenic cells and fibroblasts

Immunostaining of muscle-derived cells after 7 days in culture (the 'before-sort' condition) showed proliferation (indicated by nuclear expression of Ki67) in cells of both myogenic (desmin$^+$, and some with nuclear MyoD) and fibroblast (TE-7$^+$) phenotypes (Fig. 2A–C). Myogenic cells never expressed TE-7 and fibroblasts never expressed desmin or MyoD, confirming the divergent lineage of these cells. At 1 week post isolation, adherent cell yields showed a good correlation with the mass of the tissue sample (Fig. 2D). This cell yield is within the usual range reported by other authors for humans (Bonavaud et al., 1997; Zheng et al., 2007) and mouse (C57BL/6) hindlimb muscle (Danoviz and Yablonka-Reuveni, 2012).

To reliably obtain human myogenic cell populations with less than 5% non-myogenic cells, two passes through a MACS (magnetic-activated cell sorting) column selecting for CD56$^+$ cells were required. Fig. 2E shows the cell yields for each of the sorting steps from seven biopsies with variable initial cell numbers. The cell composition of the 'before sort' and sorted fractions of a typical experiment are shown in Fig. 2F–J. As can be seen in Fig. 2G–J, a high incidence of Ki67 expression persisted post-sorting in both main cell populations (86±5% in CD56$^+$/desmin$^+$ and 89±4% in CD56$^-$/TE-7$^+$ cells, n=5 subjects). The proportion of these major cell types (myogenic and non-myogenic, respectively) in the before-sort population also matches published data for both human muscle (Stewart et al., 2003) and mouse muscle (Bosnakovski et al., 2008).

The sort-1 CD56$^-$ fraction was a highly enriched TE-7$^+$ fibroblast population (91±3%, n=5; Fig. 2F,H), with the remaining cells being myogenic (CD56$^+$/desmin$^+$; Fig. 2F). After 2 days, the greater proliferation rate of fibroblasts in this fraction had produced a 95±4% TE-7$^+$ fibroblast population (n=4 subjects); this purity increased with time in culture (data not shown). The double sorting resulted in very high purity (97±1%, n=5 biopsies) of the sort-2 CD56$^+$ population (Fig. 2J). In serum-free myogenic differentiation medium (without insulin or transferrin), CD56$^+$ and desmin$^+$ cells fused to form large myotubes, which strongly expressed MHC (Fig. 2M), confirming that CD56 expression identifies myogenic cells from human skeletal muscle, which can complete terminal myogenic differentiation. In contrast, the sort-1 CD56$^-$/TE-7$^+$ population showed no capacity for myogenic differentiation under the same conditions (Fig. 2N). In highly confluent conditions, CD56$^-$ cells deposited an intricate TE-7$^+$ fibrotic extracellular matrix (supplementary material Fig. S4), which is a defining characteristic of fibroblasts (Sabatelli et al., 2001).

Presence of putative adipogenic stem cell subsets from human muscle identified by CD15 and PDGFRα expression

In sort-2 CD56$^+$ cultures, expression of the CD15 antigen was extremely rare and was always <1% of any population (supplementary material Fig. S5A,B). CD15 was always co-expressed with CD56 confirming the myogenic identity of these cells (supplementary material Fig. S5C). No evidence of CD15 expression was found in the CD56$^-$/TE-7$^+$ cells under any condition. Analysis of mixed populations of cells demonstrated that nearly all CD56$^-$ cells showed strong staining for PDGFRα, whereas this marker was almost undetectable on CD56$^+$ cells (supplementary material Fig. S5D).

Fibroblasts vastly expand intracellular lipid stores in response to fatty acid treatment

In the untreated control condition, CD56$^+$/desmin$^+$ cells showed very few lipid droplets (Fig. 3A, top row, B), whereas there was significantly greater Oil-Red-O staining in the CD56$^-$/TE-7$^+$ cells (Fig. 3A, third row, C). To assess the adipogenic response of muscle-derived cells to a physiologically relevant nutrient challenge, cultures were exposed to a physiological dose and ratio of saturated to unsaturated FAs complexed to serum albumin. CD56$^+$ cells and CD56$^-$ cells responded very differently. After 72 hours, the CD56$^-$/TE-7$^+$ cells had accumulated large amounts of lipid (Fig. 3A, bottom row), such that their cytoplasm was almost completely filled with Oil-Red-O$^+$ droplets (Fig. 3E,F). By contrast, CD56$^+$/desmin$^+$ cells accumulated significantly fewer lipid droplets over this time (Fig. 3A, second row, D,F). These different responses were conserved in mixed cultures obtained from the sort-2 CD56$^-$ fraction (Fig. 3G,H).

Adipogenic transcription factors are highly upregulated in myogenic cells and fibroblasts in response to fatty acid treatment

We tested whether sort-2 CD56$^+$/desmin$^+$ myogenic cells and sort-1 CD56$^-$/TE-7$^+$ fibroblasts expressed the master adipogenic

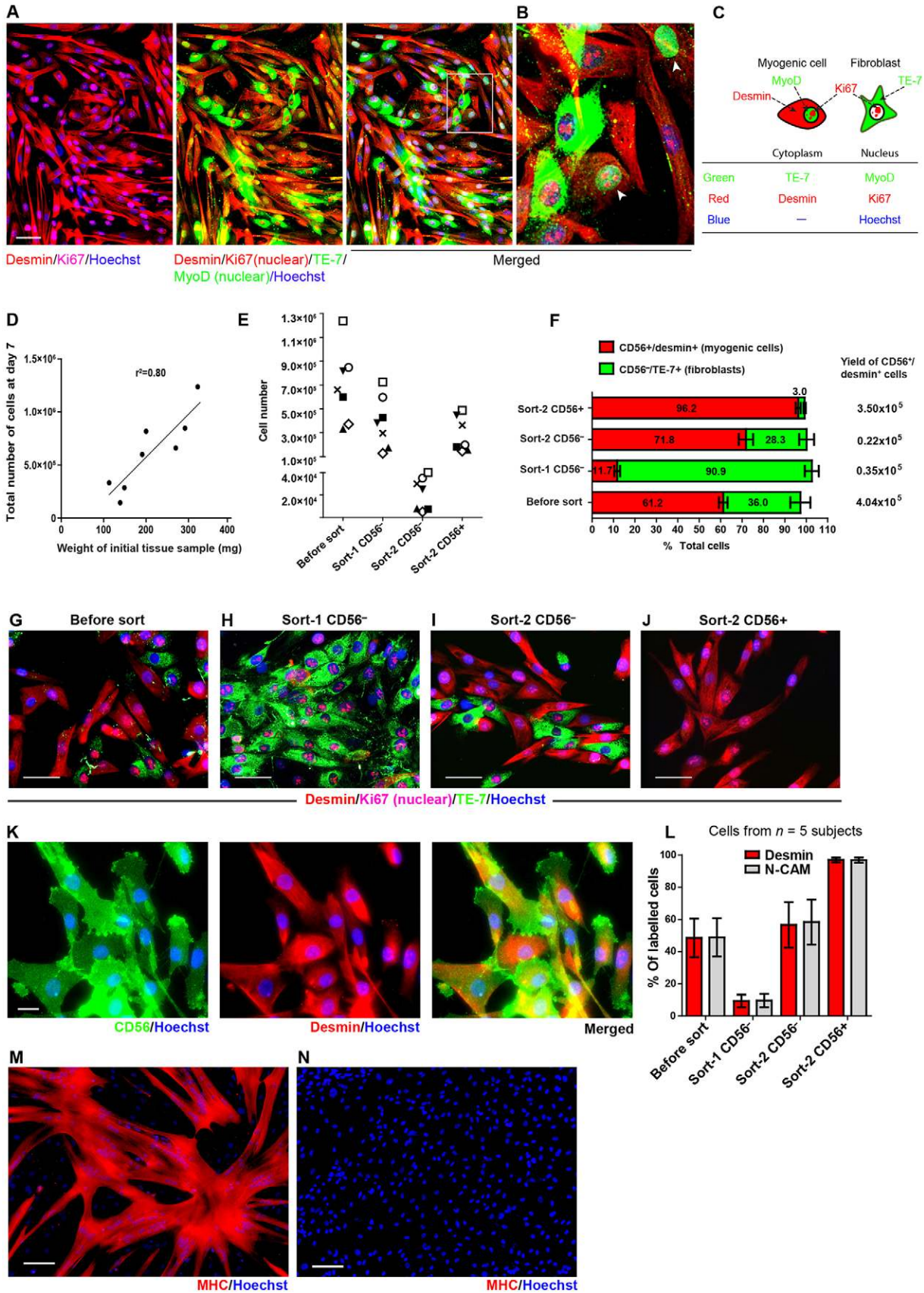

Fig. 2. See next page for legend.

transcription factors PPARγ and C/EBPα before and after treatment with FAs (Figs 4,5). Nuclear expression of these transcription factors was measured in individual cells using a highly-sensitive image analysis approach. In control conditions, nuclear PPARγ expression was readily detectable in the fibroblasts ($CD56^-$/$TE\text{-}7^+$, Fig. 4A), but was low in myogenic cells ($CD56^+$/$desmin^+$, Fig. 4D). Both cell populations showed a highly significant increase in PPARγ following 72 hours of FA treatment (Fig. 4B,C, fibroblasts; Fig. 4E,F, myogenic cells). Under control conditions, staining for nuclear C/EBPα was extremely weak in both $CD56^-$/$TE\text{-}7^+$ cells (Fig. 5A) and $CD56^+$/$desmin^+$ cells (Fig. 5D), but after 72 hours of FA treatment, C/EBPα was seen with a strong punctate nuclear localisation in the majority of cells, irrespective of their desmin or TE-7 identity (Fig. 5B,E). The increase in the measured nuclear fluorescence intensity of C/EBPα in both $CD56^-$ and $CD56^+$ populations was large and highly significant (Fig. 5C,F).

Evidence of fatty acid-induced adipogenic conversion in fibroblasts

Both $CD56^+$/$desmin^+$ and $CD56^-$/$TE\text{-}7^+$ cell types showed a significant increase in PPARγ and C/EBPα in response to FA treatment, but whereas the $CD56^+$/$desmin^+$ cells retained their original myogenic phenotype (Fig. 4D,E; Fig. 5D,E), the fibroblasts ($CD56^-$/$TE\text{-}7^+$) did not. Immunostaining of fibroblasts maintained in the fatty-acid-containing medium revealed that as the number of lipid droplets increased, the cytoplasmic TE-7 staining decreased significantly relative to untreated controls (Fig. 6, compare A with B). By 3 days, the incidence of cytoplasmic TE-7 expression had declined dramatically (Fig. 6C), with many cells adopting an adipocyte-like morphology (e.g. starting to 'round up' and display peripheral nuclei; Fig. 6D).

Fig. 2. Characterisation and quantification of cells in culture and the sorting process. (A) Immunostaining of the initial (pre-sort) cells after 7 days in culture showing distinct myogenic ($desmin^+$, nuclear MyoD) and fibroblast populations (high cytoplasmic TE-7) undergoing proliferation ($Ki67^+$ nuclei). (B) Enlarged view of area in A outlined by white box in merged view. MyoD expression (arrowhead) is seen only in myogenic ($desmin^+$/$TE\text{-}7^-$) cells. (C) Schematic key for immunostaining in A,B. (D) Total adherent muscle cell yields from $n=8$ biopsies once contaminating blood cells were removed, plotted in relation to the initial weight of the tissue sample. (E) Total cell yields for different stages of the MACS process applied to seven successive biopsies (different symbol for each biopsy). (F) Results of one typical cell-purification experiment starting from a biopsy of 291.8 mg, showing the cell phenotypes and yields obtained. (G) Cells before sorting (mixed composition of $desmin^+$ myogenic cells and $TE\text{-}7^+$ fibroblasts, all still mononucleate and many $Ki67^+$); (H) sort-1 $CD56^-$ cells (almost exclusively $TE\text{-}7^+$); (I) sort-2 $CD56^-$ cells (mixed composition); (J) sort-2 purified $CD56^+$/$desmin^+$ cells. (K) sort-2 purified $CD56^+$ population cells maintained for 2 weeks in culture co-express CD56 and desmin. (L) Quantification of $CD56^+$ and $desmin^+$ cells from the same cell populations plated in duplicate for each marker (data are means ± s.d.). Irrespective of sort fraction, CD56 and desmin are always expressed by the same percentage of cells in culture. (M) Sort-2 purified $CD56^+$ cells transferred to serum-free myogenic differentiation medium differentiate within 4 days into large multinucleated myotubes which express MHC. (N) No evidence of myotube formation was found in the $CD56^-$ population under serum-free conditions. Observations for M and N were made on cells derived from $n=7$ separate biopsies. Scale bars: 50 μm (A,G–J); 20 μm (K); 100 μm (M,N).

Adipogenic transdifferentiation of intramuscular fibroblasts, but not myogenic cells, cultured in AIM

The standard AIM protocol elicited very different responses in $CD56^+$/$desmin^+$ cells compared with $CD56^-$/$TE\text{-}7^+$ cells. $CD56^-$/$TE\text{-}7^+$ cells started to accumulate cytoplasmic lipid droplets within 7 days of AIM treatment, and by 15 days, the lipid load was so large in some cells that they progressively adopted a round morphology and shift of nuclear position towards the edge of the cell (Fig. 7A,B). It was necessary to measure the fluorescence intensity of the nuclear adipogenic transcription factors after 7 days in AIM rather than at full term (15 days), because at 15 days, many of these cells became so engorged with Oil Red O^+ lipid that large proportions of their nucleus (sometimes the entire nucleus) was not visible for analysis. At 7 days, a marked and significant upregulation in nuclear PPARγ expression was observed in the $CD56^-$/$TE\text{-}7^+$ cells (Fig. 7A,H).

By contrast, after 7 days in AIM, $CD56^+$/$desmin^+$ cells expressed only very low levels of PPARγ (Fig. 7E), showing a small but significant decline from the control (Fig. 7I), and failed to synthesise any additional lipid (Fig. 7C,D). Many of the $CD56^+$ cells confirmed their myogenic phenotype by proceeding to differentiate into large multinucleated myotubes (as might be expected given the low serum content of the adipogenic nutrition medium), strongly expressing MHC and nuclear myogenin (Fig. 7F). In addition, all myotubes maintained very strong CD56 expression (Fig. 7G), demonstrating the usefulness of the CD56 antigen as a myogenic marker throughout all phases of human *in vitro* myogenesis, and confirming a lack of adipogenic transdifferentiation in this population.

In the $CD56^-$/$TE\text{-}7^+$ cells, lipid accumulation was coincident with a marked increase in C/EBPα expression after 7 days of AIM treatment (Fig. 8A,B,K). Although an increase in lipid content was very clear at this time, the cells still contained cytoplasmic TE-7 (Fig. 8A,B,E,F), confirming their fibroblast origin. However, by 15 days, intracellular TE-7 had been extruded as these cells made the transition into adipocytes (Fig. 8C,D,G,H). The same response was seen for another fibroblast marker, collagen VI (supplementary material Fig. S6). By contrast, $CD56^+$/$desmin^+$ cells failed to upregulate C/EBPα, even after 15 days in AIM (Fig. 8I,J,L).

Extremely rare $CD15^+$ cells are not the primary adipogenic precursors in adult human skeletal muscle

When treated with FAs, the few $CD56^+$/$CD15^+$ cells present often exhibited a distinct morphology and accumulated variable amounts of lipid as small droplets (supplementary material Fig. S7A), although this adipogenic response to FA treatment was far less marked than that achieved by the $TE\text{-}7^+$ fibroblast population (supplementary material Fig. S7B). After 7 days in AIM, $CD15^+$ cells were still very rare and responded by synthesising limited amounts of lipid (supplementary material Fig. S7A). At 15 days, they had adopted a variety of morphologies, often with a single long tail-like projection (supplementary material Fig. S7D,E). However, even on long-term AIM treatment, these $CD15^+$ cells maintained their $CD56^+$ expression (supplementary material Fig. S7D,E), suggesting that they are descendents of $CD56^+$ cells which remain committed to the myogenic lineage even under conditions which strongly favour adipogenesis. CD15 is therefore unlikely to be of use as a surface marker to identify adipogenic precursors in human skeletal muscle.

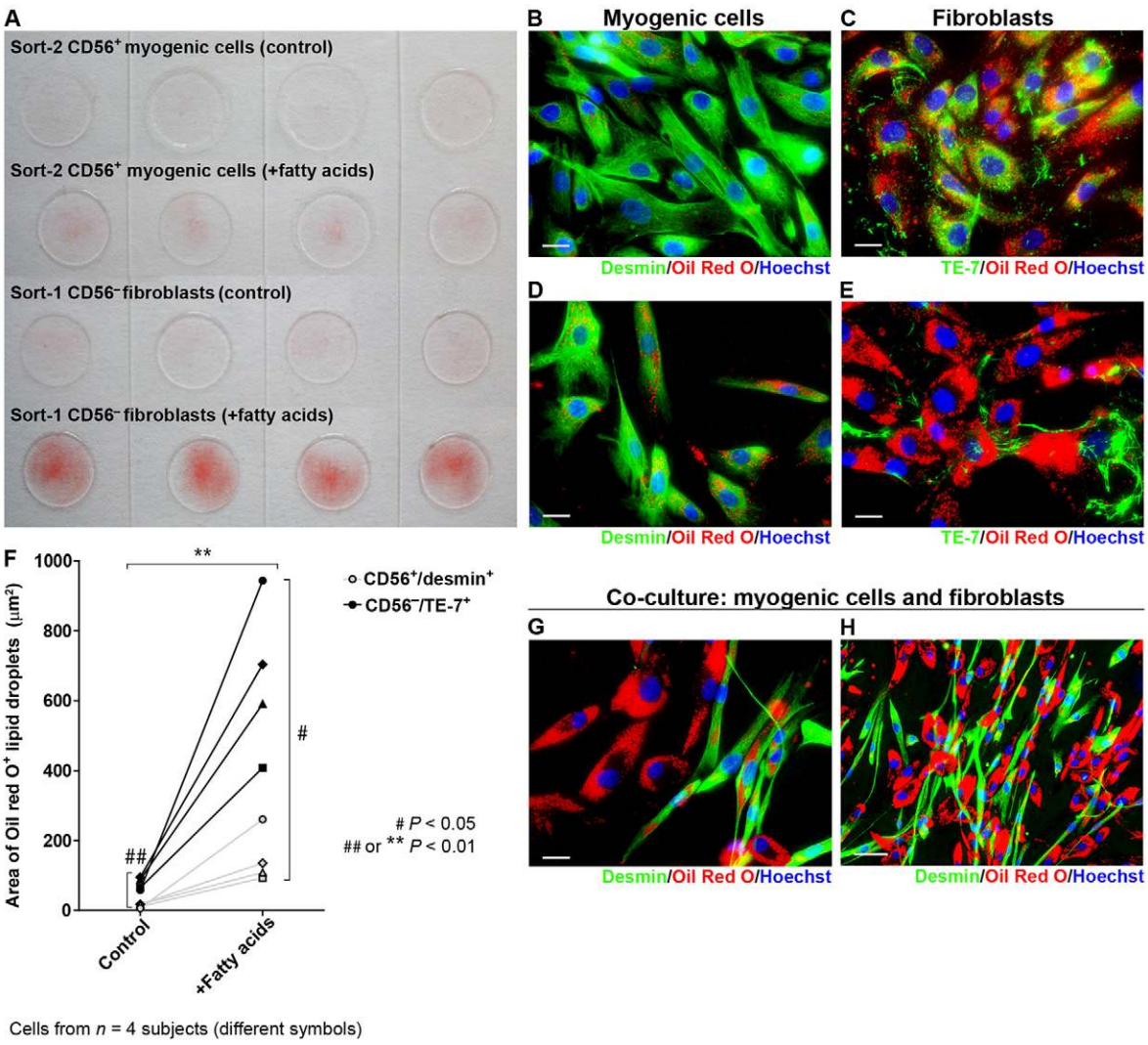

Fig. 3. Effect of FA treatment on muscle-derived cells in culture. (A) Replicate wells (n=4) containing the same density of purified sort-2 CD56$^+$ cells (top two rows) and CD56$^-$ first sort CD56$^-$ cells (bottom two rows) either exposed to FA treatment or left untreated (vehicle-only negative controls), fixed after 3 days and then stained with Oil Red O. The difference in Oil Red O staining between the two populations is evident to the naked eye (compare row 2 with row 4). (B–E) CD56$^+$/desmin$^+$ cells and CD56$^-$/TE-7$^+$ cells before (B,C) and after (D,E) treatment with FAs. (E) After FA treatment the CD56$^-$/TE-7$^+$ cells acquired many lipid droplets and downregulated their TE-7 expression. (F) Mean area per cell of Oil-Red-O$^+$ lipid droplets in CD56$^+$/desmin$^+$ and CD56$^-$/TE-7$^+$ cultures before and after FA treatment, determined from independent experiments on n=4 biopsies (individual biopsies represented by the same symbol); , main effect of fat treatment; #, comparison between cell types (mixed-model repeated measures ANOVA). (G,H) Co-culture population of myogenic cells and fibroblasts. (G) Myogenic CD56$^+$/desmin$^+$ cells accumulate very little lipid, whereas the non-myogenic CD56$^-$/TE-7$^+$ cells have acquired large numbers of lipid droplets in their cytoplasm. (H) Lower-power view to show that only, and all, non-myogenic cells are highly adipogenic. Scale bars: 20 μm (A–G); 50 μm (H).

Discussion

An increase in intramuscular adipocytes is a characteristic feature of obesity, type-2 diabetes, sarcopenia and muscular dystrophies, and is closely associated with systemic metabolic dysregulation (Goodpaster et al., 2000; Hegarty et al., 2003), cardiovascular risk (Yim et al., 2007) and functional impairment (Nawrocki and Scherer, 2004; Marcus et al., 2012; Tuttle et al., 2012), yet the source of these adipocytes remains equivocal. To investigate the cellular origin of adipose tissue in human skeletal muscle, we optimised a double immuno-magnetic cell-sorting protocol for the efficient purification of myogenic (CD56$^+$) and non-myogenic (CD56$^-$) cells from muscle-derived cultures in order to independently examine their adipogenic potential. Our main finding was that only sort-1 CD56$^-$ cells, which consisted almost exclusively of fibroblasts (Fig. 2G), showed robust adipogenic potential. Our results demonstrate that fibroblasts are the main source of adipocytes in human-muscle-derived cultures.

Characterisation of cell types

Early immunostaining of unsorted human-muscle-derived cells at 72 hours post isolation revealed that these cultures consist predominantly of myogenic cells and fibroblasts (supplementary material Figs S2, S3). At 7 days post-isolation these populations persisted with almost all adherent cells in cultures belonging to either the myogenic (CD56$^+$/desmin$^+$) or fibroblast (CD56$^-$/TE-7$^+$) lineages (Fig. 2F). Double immuno-magnetic purification of

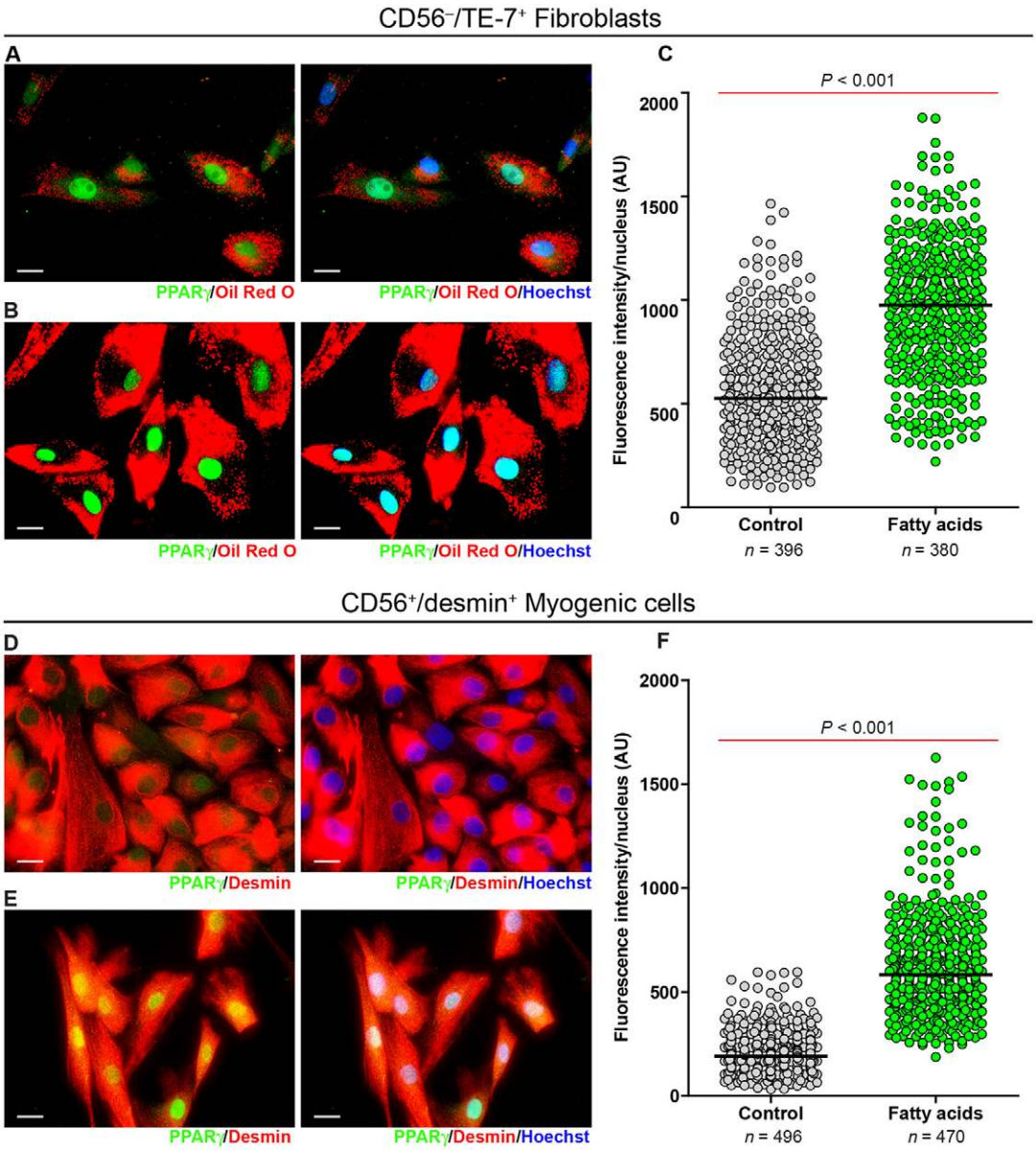

Fig. 4. PPARγ expression in response to FA treatment. PPARγ in CD56^{-}/TE-7^{+} fibroblasts before (A) and after (B) FA treatment. Within the non-myogenic TE-7^{+} population, PPARγ immunoreactivity was present even in the control condition, but still rose significantly following FA exposure. (C) Quantification of nuclear PPARγ fluorescence intensity in individual nuclei shows significant upregulation after FA treatment. PPARγ expression in CD56^{+} myogenic cells before (D) and after (E) treatment with FAs. The myogenic cells had very low nuclear PPARγ staining under proliferation conditions, but markedly upregulated this following the addition of FA; yet still maintained their desmin expression and myogenic morphology. Scale bars: 20 µm. (F) Quantification of nuclear PPARγ fluorescence intensity in individual nuclei shows significant upregulation after FA treatment. Horizontal black lines in C and F indicate the median. Data were compared using the Mann–Whitney U-test.

CD56^{+} cells produced exclusively myogenic cultures which highly expressed desmin and proliferated well (Fig. 2J,K). Upon serum withdrawal, CD56^{+}/desmin^{+} cells readily completed terminal differentiation to form large multinucleated myotubes expressing MHC (Fig. 2M), confirming CD56 as a useful marker for human primary myogenic cells as suggested by others (Cashman et al., 1987; Moore et al., 1987; Schubert et al., 1989; Illa et al., 1992; Mackey et al., 2009). By contrast, the CD56^{-} cells obtained from sorting expressed the fibroblast specific antigen TE-7 (Goodpaster et al., 2008) in culture and were incapable of myogenic differentiation (Fig. 2N). Our data are thus in firm agreement with Stewart and co-workers (Stewart et al., 2003), who found that CD56 and desmin labelled the same percentage of cells in human-muscle-derived cell populations, and that the vast majority of remaining cells expressed the fibroblast marker TE-7. In our study, almost every cell in this CD56^{-} fibroblast population also stained strongly for PDGFRα, which has previously been shown to be a marker for connective tissue fibroblasts (Olson and Soriano, 2009; Murphy et al., 2011), as well as a marker for FAPs in mouse skeletal muscle (Uezumi et al., 2010; Uezumi et al., 2011). In human muscle, however, we show that PDGFRα is expressed in a distinct punctate pattern by virtually all connective

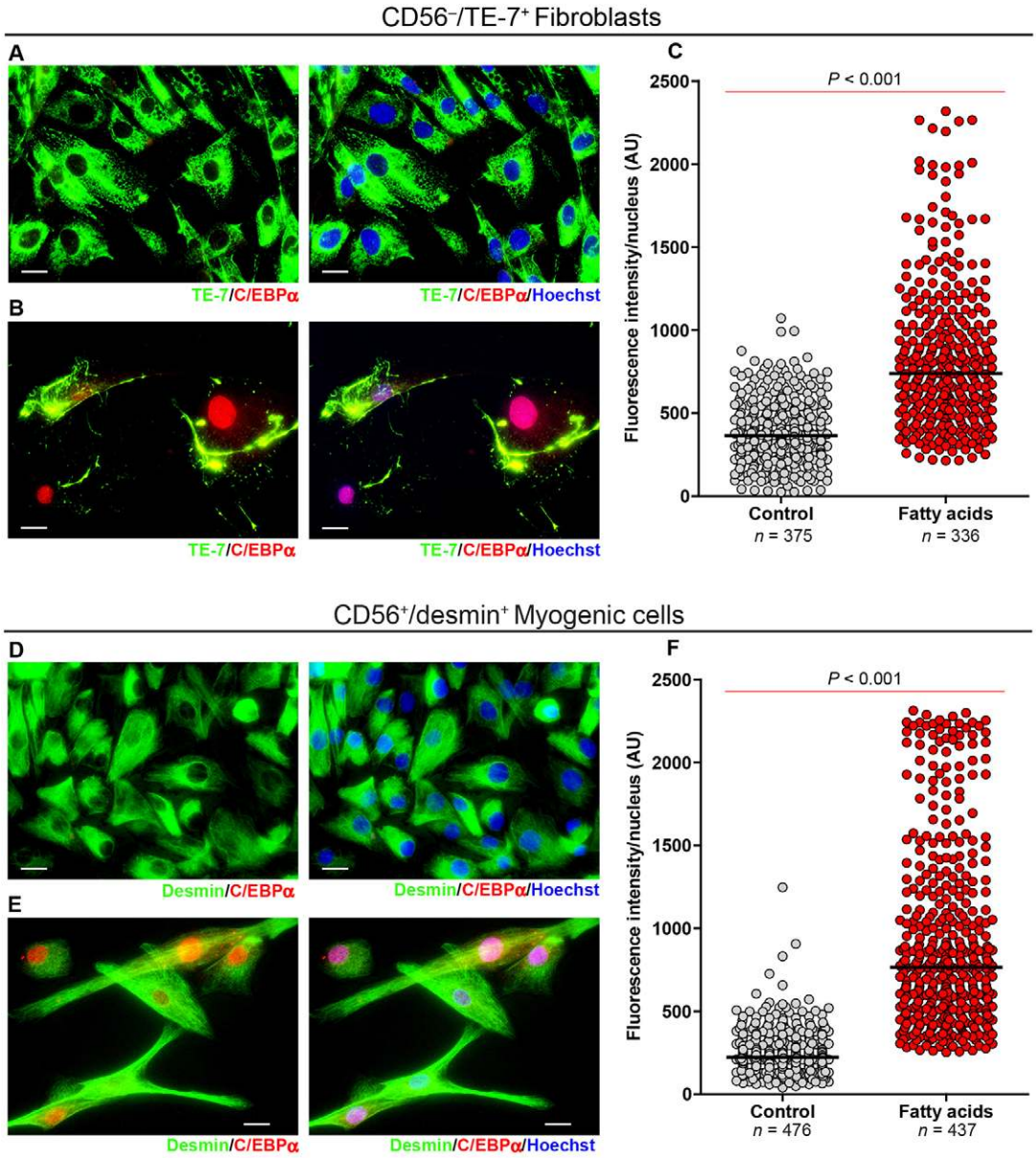

Fig. 5. C/EBPα expression in response to FA treatment. C/EBPα in CD56^{-}/TE-7^{+} fibroblasts before (A) and after (B) FA treatment. Within the non-myogenic TE-7^{+} population, expression of C/EBPα was very low in proliferating fibroblasts, but subsequent to FA stimulation, very strong nuclear localisation of this antigen appeared in most cells. C/EBPα expression in CD56^{+}/desmin^{+} myogenic cells before (D) and after (E) FA. The myogenic cells had extremely low nuclear C/EBPα staining under proliferation conditions, but markedly upregulated this following the addition of FAs, despite maintaining desmin expression and myogenic morphology. Scale bars: 20 µm. (F) Quantification of nuclear C/EBPα fluorescence intensity in individual nuclei shows significant upregulation after FA treatment in both cell types. Horizontal black lines in C and F indicate the median. Data were compared using the Mann–Whitney U-test.

tissue-producing fibroblasts as well as by a very small population of myogenic cells (3.67±0.86%) (supplementary material Fig. S2E; Fig. S3D).

A variety of other cell types with stem cell properties might be expected to be present in the original population of cells obtained by dissociation; these include haematopoietic stem cells, endothelial cells and various muscle-derived stem cell fractions identified in mouse muscle, and a few in human muscle. Typically, the other muscle ‘stem’ cell types are very small fractions of the population obtained after muscle dissociation, and in several cases only a subfraction of them may be capable of alternative lineage differentiation (Yin et al., 2013). For example, Penton and colleagues (Penton et al., 2013) recently examined the ‘side population’ (SP) cells obtained from normal mouse muscle by FACS. The SP cells are <3% of the total cell population obtained by dissociation; about 80% of them are endothelial cells, 2–5% are haematopoietic and about 5% are the ‘lineage-negative’ SP cells that were shown to be able to adopt a fibro-adipogenic fate in subsequent culture. Endothelial and haematopoietic cells do not give rise to adipocytes (Berry and Rodeheffer, 2013), and our cell counts showed that the CD56^{-}/TE-7^{-} population of cells (which might include these cells) is also far too small to account for the number of adipocytes observed in our cultures. The non-adherent nature of some of

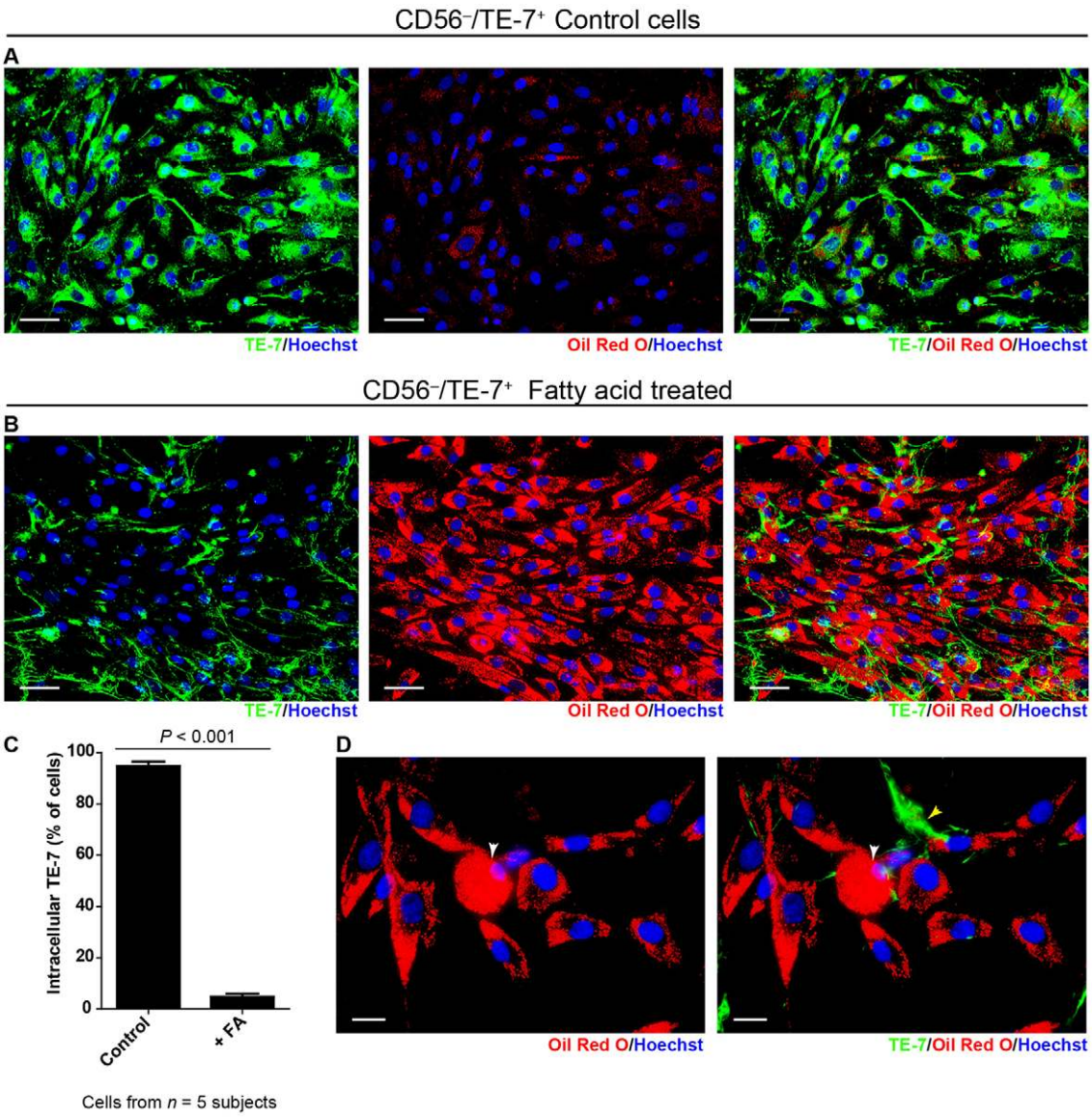

Fig. 6. Effect of FA treatment on TE-7 expression in CD56⁻ cells in culture. (A) In the control condition all cells have a fibroblast phenotype, expressing TE-7 in the cytoplasm as well as depositing it extracellularly on the substrate, and contain some small lipid droplets. (B) After treatment with FA their lipid content is hugely increased in co-incidence with a reduction in intracellular TE-7 immunoreactivity. (C) Quantification of the significant decrease in cytoplasmic TE-7 in human muscle fibroblasts treated with FA; data here are means ± s.d. and were analysed using an unpaired *t*-test. (D) FA treatment induced the synthesis of large amounts intracellular triglyceride in fibroblasts. As the lipid load increased, these cells started to become more spherical (white arrowhead) and released their TE-7⁺ protein in fibrous form (yellow arrowhead). Scale bars: 50 μm (A,B); 20 μm (D).

these cell types also ensures they would have been discarded before obtaining the sorted cells – this applies to the 'small refractory' cell population identified by Meng and co-workers (Meng et al., 2011), the 'muscle-derived stem cells' (Lee et al., 2000; Qu-Petersen et al., 2002) and the pericytes identified in human muscle by Dellavalle and colleagues (Dellavalle et al., 2011). The small refractory cells (Meng et al., 2011) were initially both $CD56^{-}$ and non-adherent; only after 14 days in culture and when provided with collagen-I substrate were they able to adhere, and eventually gave rise to some $CD56^{+}$ cells. The 'muscle-derived stem cells' obtained by Lee and co-workers (Lee et al., 2000), could only be obtained by repeated subculturing of non-adherent cells, and the $CD56^{-}$ 'pericytes' with multi-lineage potential that were identified in human muscle (Dellavalle et al., 2011) are also a small fraction (estimated at about 2–4% of the isolated cell population). These cells were poorly adherent, requiring a collagen substrate (or, in some cases, Matrigel) to adhere and grow. Our adipogenic cells were very adherent within 72 hours of dissociation, even on a plastic substrate, so are clearly very different.

Of the potential contaminating stem or progenitor cells which are $CD56^{+}$, we specifically looked for the adipogenic $CD15^{+}$ population (Pisani et al., 2010), but these were extremely rare, accumulated much less lipid than the fibroblasts and always co-expressed CD56 (confirming their myogenic identity), so these can also be rejected as the source of our adipogenic cells.

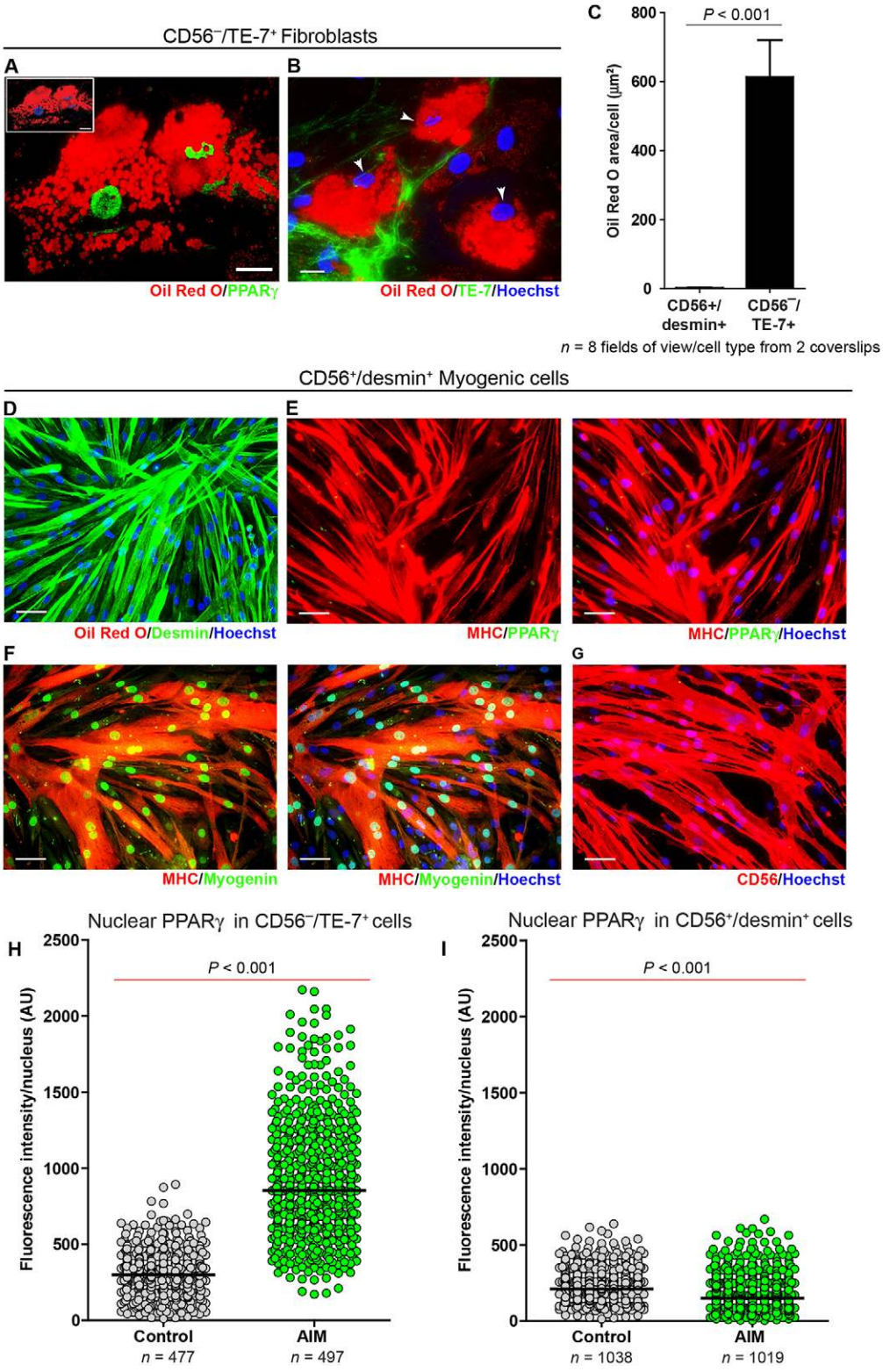

Fig. 7. Lipid content and PPARγ expression in response to AIM treatment. (A) After 15 days in AIM many of the CD56$^-$ cells had differentiated into adipocytes, highly expressing nuclear PPARγ and containing very large lipid droplets. Insert in A shows Oil Red O and blue Hoechst$^+$ nuclei. (B) Following AIM treatment, CD56$^-$/TE-7$^+$ cells were more rounded and in many the nucleus had shifted towards the periphery of the cell (arrowheads). (C) After 15 days, the lipid content of CD56$^-$ cells was markedly higher than in CD56$^+$ cells; data are means ± s.d. (D) CD56$^+$/desmin$^+$ cells accumulated very little lipid and (E) did not upregulate PPARγ. (F) The CD56$^+$/desmin$^+$ cells differentiated into myotubes expressing MHC and nuclear myogenin (G) and retained their CD56 surface marker. Scale bars: 20 μm (A,B); 50 μm (D–G). Quantification of nuclear PPARγ fluorescence intensity levels showed a marked and significant increase in the expression of this transcription factor in CD56$^-$/TE-7$^+$ cells after 7 days in AIM (H), but a tiny though significant decrease in CD56$^+$ cells (I). Horizontal black lines in H and I indicate the median. Data were compared using the Mann–Whitney U-test.

Myoendothelial cells are reported to be CD56$^+$, but are also very rare, and grow preferentially in endothelial-specific growth medium on collagen-I-coated plates (Zheng et al., 2007). This is completely different from our pre-sort conditions (plastic plates and a muscle-specific proliferation medium), and therefore we consider that even if present initially they would make no significant contribution to our CD56$^+$ population. It is for quantitative reasons that we attribute the observed adipogenic response seen in our cultures to the fibroblasts, and not to myogenic cells or any other cell types. In our cultures, the CD56$^-$/TE-7$^-$ population (which includes most of the contaminating stem cell types) was very small and the CD56$^+$

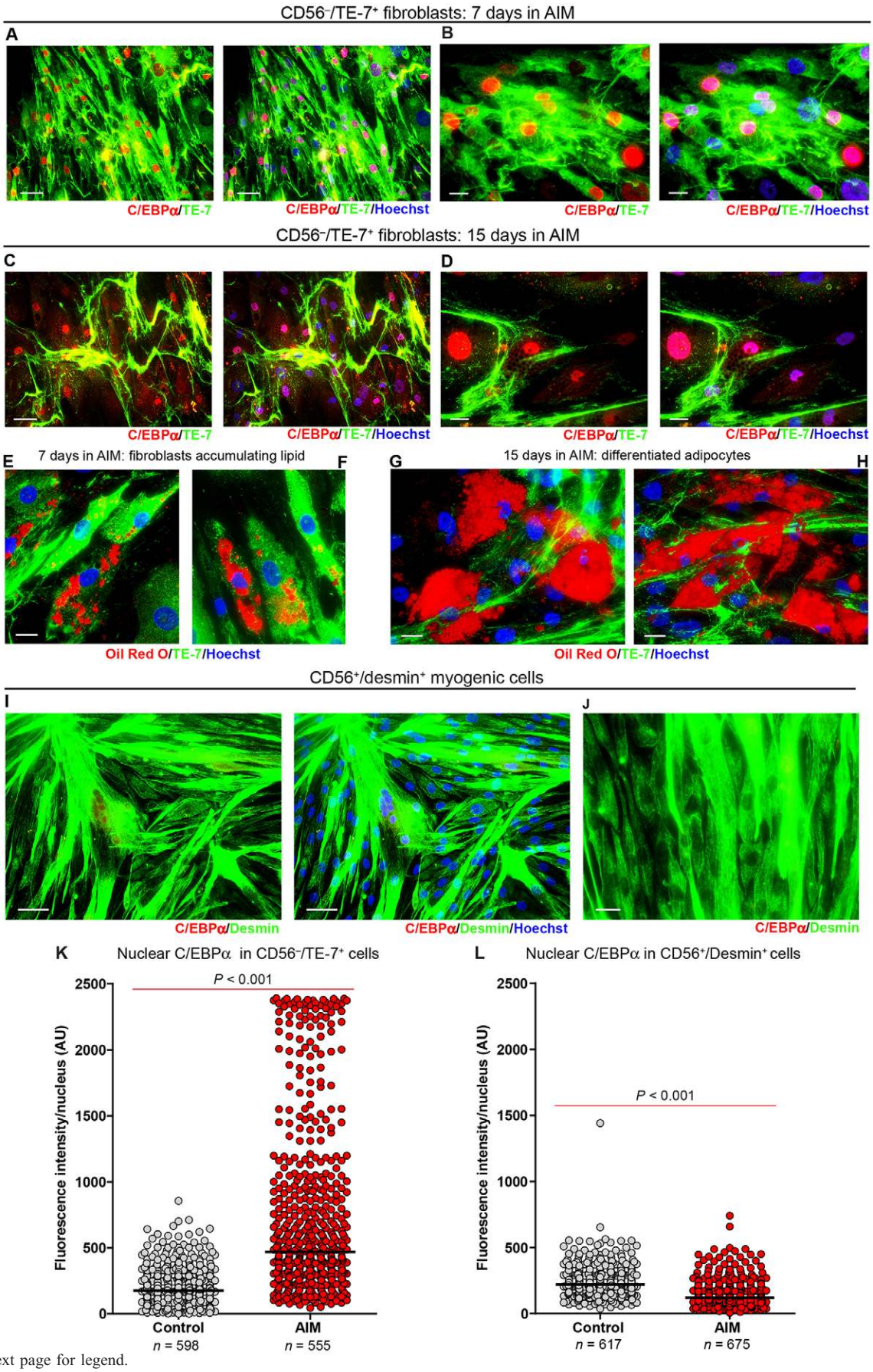

Fig. 8. See next page for legend.

minor cell types also too few (and insufficiently adipogenic). There here was no significant expansion of $CD56^-/TE\text{-}7^-$ cell types during culture, and most importantly, the adipogenic response was seen in almost all fibroblasts, not just in a small subfraction of them. The extent of the adipogenic response demonstrated by our abundant (confirmed) fibroblast population simply far outstrips anything that very small populations of other cell types with potential for adipogenic differentiation could have been achieved in the time available in culture.

Testing the adipogenic potential of purified cell populations

The purified cell populations were examined for their response to two adipogenic stimuli: treatment with FA and AIM, and our main findings are summarised in supplementary material Fig. S9. To identify changes in the essential adipogenic transcription factors PPARγ and C/EPBα, we used a highly sensitive image analysis approach capable of detecting and quantifying low levels of nuclear expression on a cell-by-cell basis. Using this technique, the full range of variation in protein expression between individual nuclei could be measured – a significant improvement over manual counting or assessment of total or nuclear extracts.

FA treatment induced a strong upregulation of PPARγ and C/EBPα expression in the fibroblasts, and an extensive accumulation of lipid droplets after only 3 days' treatment (Fig. 3; Fig. 4A–C). Simultaneously, these cells could be seen to downregulate and extrude their fibroblast marker (TE-7) (Fig. 6). Long-chain FAs are known ligands for PPARγ and can induce adipogenesis in preadipocytes located in white adipose tissue (Amri et al., 1995). Our finding that $TE\text{-}7^+$ fibroblasts contain lipid droplets, constitutively express PPARγ under normal proliferative conditions and readily accumulate intracellular triglyceride in response to FA treatment, suggest they have an inherent ability for adipogenic conversion.

The adipogenic potential of the muscle fibroblasts ($CD56^-/TE7^+$) was further confirmed by treatment with AIM. These cells showed a vast accumulation of lipid, typical adipocyte morphology, high expression of both PPARγ and C/EBPα and downregulation of cytoplasmic TE-7 (Figs 7,8). By 15 days, evidence of complete adipogenic differentiation was apparent (Fig. 7A,B; Fig. 8G,H). In addition to their role in intramuscular adipogenesis, the *in situ* localisation of TE-7 in elderly human muscle (Fig. 1E; supplementary material Fig. S4B) indicates a role for $TE\text{-}7^+$ fibroblasts in connective tissue deposition and fibrosis. Furthermore, $CD56^-$ fibroblasts cultured long term at confluence produced an extensive fibrotic extracellular matrix (supplementary material Fig. S4A). The observation that human muscle fibroblasts highly express collagen VI (supplementary material Fig. S6) suggests a role for these cells in the pathogenesis of Ullrich muscular dystrophy, Bethlem myopathy and myosclerosis, in which collagen VI is mutated (Zou et al., 2008; Bönnemann, 2011).

Interestingly, the $CD56^+$ cells also responded to FA treatment by a strong induction of nuclear C/EBPα expression and upregulation of PPARγ, but maintained both normal morphology and expression of desmin and accumulated only limited amounts of lipid. By contrast, when $CD56^+$ myogenic cells were exposed to the AIM they failed to upregulate expression of either PPARγ or C/EBPα, and did not accumulate lipid droplets. They showed a strong retention of their myogenic phenotype, with many completing normal terminal differentiation into myotubes expressing nuclear myogenin and MHC. This result using human myogenic cells parallels the resistance of mouse satellite cell progeny to adipogenic transdifferentiation seen by Starkey and colleagues (Starkey et al., 2011).

Muscle-derived stem cells expressing the CD15 antigen have been proposed to have a high propensity for adipogenic conversion. Pisani and colleagues (Pisani et al., 2010) identified a sub-population of bipotent $CD56^+/CD15^+$ cells among an already small population of 'muscle stem cells' from human muscle. This bipotent population was found to be capable of giving rise to either $CD56^+/CD15^-$ myogenic cells, or $CD56^-/CD15^+$ adipogenic cells. In our experiments, $CD15^+$ cells were extremely rare (<1%) and always co-expressed the myogenic marker CD56 (supplementary material Fig. S5). In response to treatment with FAs and AIM, $CD15^+$ cells accumulated some lipid and also exhibited morphologies distinct from the $CD56^+/CD15^-$ and $CD56^-/TE\text{-}7^+$ cells, but showed no evidence of adipogenic differentiation (supplementary material Fig. S7). This does not preclude the involvement of $CD15^+$ cells in either adipogenesis or myogenesis, but their very low abundance indicates they are not a major contributor to either lineage.

Role of the transcriptional activators C/EBPα and PPARγ

The transcriptional activators C/EBPα and PPARγ are both necessary and sufficient for the induction of adipogenic differentiation (Gregoire et al., 1998; Kirkland et al., 2002). During adipogenesis, PPARγ and C/EBPα positively regulate each other's expression and in doing so, activate the large array of genes required to produce and maintain the mature adipocyte phenotype (Rosen et al., 2002; Tang and Lane, 2012). Data derived from both FA and AIM treatment confirm roles for C/EBPα and PPARγ in the terminal differentiation of muscle-derived $CD56^-/TE\text{-}7^+$ fibroblasts to adipocytes. However, our data also indicate a wider metabolic role for both PPARγ and C/EBPα that is independent of adipogenesis *per se.* When treated with FAs, our human $CD56^+$/desmin$^+$ cells accumulated some lipid and upregulated both C/EBPα and PPAR-γ, but did not adopt an adipocyte-like morphology or lose the myogenic protein desmin. This indicates a lack of lineage alteration as the

Fig. 8. C/EPBα and TE-7 marker expression in response to AIM treatment. (A–D) C/EBPα expression in $CD56^-/TE\text{-}7^+$ fibroblasts after 7 days and 15 days in AIM. (A,B) After 7 days in AIM, almost all fibroblasts demonstrated strong intracelluar TE-7 staining and showed intense nuclear expression of C/EBPα. (C,D) At 15 days, nuclear C/EBPα expression was still high in the fibroblasts but most TE-7 staining was filamentous and extracellular. (E,F) At 7 days, many $TE\text{-}7^+$ cells making the transition into adipocytes can be seen; two different high-power examples are shown. (G,H) After 15 days in AIM, many $CD56^-$ fibroblasts had extruded the majority of their $TE\text{-}7^+$ protein and differentiated into adipocytes engorged with lipid; two different high-power examples are shown. (I,J) After 7 days (I) and 15 days (J) $CD56^+$ (myogenic) cells expressed very low C/EBPα, maintained their myogenic morphology and marker expression, and many completed differentiation into myotubes. (K,L) Quantification of nuclear C/EBPα fluorescence intensity showed a significant increase in the expression of this transcription factor in $CD56^-/TE\text{-}7^+$ cells (K), but a slight but significant decrease in $CD56^+$ cells (L). Scale bars: 50 μm (A,C,I); 20 μm (B,D–H,J). Horizontal black lines in K and L indicate the median. Data were compared using the Mann–Whitney U-test.

rearrangement of cytostructure is necessary for adipogenesis (Spiegelman and Farmer, 1982; Gregoire et al., 1998). Even myogenic cells with the highest expression of PPARγ, equivalent to that in the fibroblast population, remained phenotypically myogenic (Fig. 4E). The different responses of myogenic cells and fibroblasts to FA and AIM treatment are summarised in supplementary material Fig. S9. In effect, the response of the myogenic cells to FA and AIM treatment models modest intramyocellular lipid deposition, whereas the fibroblasts model true adipogenesis in which new adipocytes are deposited in muscle.

Retrovirus-driven overexpression of PPARγ and C/EBPα in the immortalised murine G8 myoblast cell line has been reported to induce transdifferentiation of these cells to adipocytes (Hu et al., 1995). However, even though the physiological stimulus of FA treatment that we used did upregulate these transcription factors in human primary myogenic cells, it did not result in their transdifferentiation. Thus, although the transcriptional partnership of PPARγ and C/EBPα drives adipogenic differentiation in some systems (when constitutively expressed at supraphysiological levels), these factors do not seem to induce a myogenic-to-adipocyte switch in human primary myogenic cells. The presence of PPARγ and C/EBPα alone is therefore not definitive evidence of terminal adipogenic differentiation.

Using TCF7L2 expression as a specific *in vivo* (but not necessarily *in vitro*) marker of connective tissue muscle fibroblasts (Mathew et al., 2011), Kardon and colleagues elegantly demonstrated that these cells (which are ubiquitous in skeletal muscle) are crucial for supporting muscle regeneration (Murphy et al., 2011). Cell-autonomous ablation of these cells in mouse skeletal muscle, induced by TCF7L2 driving expression of diphtheria toxin, impaired satellite cell function and subsequent muscle regeneration following injury (Murphy et al., 2011). Since intramuscular adipocytes were shown to inversely correlate with insulin sensitivity (Goodpaster et al., 2000), intramuscular fibroblasts, which give rise to adipocytes as demonstrated here, could be an important therapeutic target in insulin-resistant disorders. Conversely, it has been argued that intramuscular adipocytes might mediate the action of insulin-sensitising drugs (Sears et al., 2009).

Given that muscle fibroblasts readily increased their lipid content, upregulated PPARγ and C/EBPα, relinquished production of a connective tissue protein and differentiated into adipocytes, we conclude that these cells are the source of adipocytes in cultures derived from human skeletal muscle. This makes them a likely candidate for explaining the fibro-fatty degeneration seen in numerous pathophysiological conditions affecting skeletal muscle. Furthermore, this study provides evidence that even under conditions that strongly favour adipogenesis, $CD56^{+}$ cells cultured from human skeletal muscle do not differentiate into adipocytes, but are faithful myogenic cells. This is in agreement with recent genetic-lineage tracing of satellite cells in the mouse (Starkey et al., 2011), and data from studies on satellite-cell ablation in adult mouse muscle where muscle regeneration was completely prevented and yet fat and fibrosis were still clearly apparent (Lepper et al., 2011; Sambasivan et al., 2011).

Some fibroblast populations are reported to exhibit little to no adipogenic capacity (Wan et al., 2006). Indeed, whereas some human orbital fibroblasts are readily differentiated into adipocytes in response to stimulation by PPARγ ligands, a subset was identified as 'anti-adipogenic'. These were found to release an unidentified paracrine or chemokine factor(s) capable of regulating the adipogenic differentiation of other fibroblasts and 3T3-l1 preadipocytes *in vitro*, probably by preventing binding of PPARγ to its consensus sequence (Jeong et al., 2010; Lehmann et al., 2010). However, the differentiation potential of dermal fibroblasts has been compared to adipose-derived (mesenchymal) stem cells by Jääger and Neuman (Jääger and Neuman, 2011). Although slightly delayed in their expression of adipogenic transcription factors such as CEBPα and FABP4, the fibroblasts were capable of adipogenic differentiation, which strongly supports our contention that muscle fibroblasts possess lineage plasticity, and raises the possibility that this property also exists in fibroblasts from other tissues with, perhaps, additional lineage fates.

In conclusion, our data add to the understanding of the cellular source of adipose tissue in skeletal muscle because we show that the majority of local fibroblasts are readily capable of adipogenesis. Although our results do not exclude a contribution from satellite cells or other stem cell sub-populations to intramuscular adipose tissue *in vivo*, they do challenge the concept that they are the major or only sources. Dissection of the mechanisms underpinning the adipogenic transdifferentiation of these muscle fibroblasts will have important implications for the understanding and treatment of many human conditions where fat accumulation and fibrosis in skeletal muscle impairs regeneration, metabolism and contractile function.

Materials and Methods

Cell isolation

Using the needle biopsy technique (Bergström, 1962) with additional suction, muscle samples (range 113–330 mg; 210.5 mg ± 78.7; mean ± s.d.) were obtained from the vastus lateralis of healthy young adult male volunteers (age 25.3±3.4 years; height, 179.6±5.6 cm; mass, 71.7±9.52 kg) who gave written informed consent to participate in this study (n=14). All studies were performed with UK National Health Service Ethics Committee approval (London Research Ethics Committee; reference: 10/H0718/10) and in accordance with the Human Tissue Act and Declaration of Helsinki.

Visible adipose or connective tissue was removed from the muscle samples which were then minced into small pieces (<1 mm^3) in basal medium (PromoCell, Heidelberg, Germany) containing collagenase B (2 mg/ml, cat no. 11 088 807 001, Roche, Germany) and dispase II (2 mg/ml, Sigma, cat. no. D4693-1G) and incubated for 1 hour at 37℃ with trituration every 15 minutes to dissociate muscle-derived cells. Enzymatic dissociation was terminated by addition of proliferation medium (C-23060; Promocell, Heidelberg, Germany) and the cell suspension passed through a 100 μm filter (BD Falcon) to remove myofibre debris. The filtered cell solution was then centrifuged at 657 ***g*** for 6 minutes at 20℃.

Cell culture

The composition of all culture media used is shown in supplementary material Table 1. The cell pellet was resuspended in a small volume of proliferation medium and transferred to a T-25 tissue culture vessel (Nunc, Germany). Cells were maintained in a humidified incubator at 37℃ and 5% CO_2 for 7 days, allowing the majority of cells to attach. The medium was replaced every 48 hours, and to avoid losing myogenic cells, non-adherent cells from the first change were collected by centrifugation, resuspended in fresh medium and returned to their original culture vessel. At day 7 the cell monolayer was rinsed with sterile phosphate buffered saline (PBS), trypsinised (0.8% Trypsin, Gibco) and counted using a haemocytometer.

Immuno-magnetic cell sorting procedure

A schematic representation of the immuno-magnetic sorting procedure is shown in supplementary material Fig. S1. We modified the procedure used by Abou-Khalil and colleagues (Abou-Khalil et al., 2009). After cells had been counted, they were centrifuged and resuspended in sterile-filtered magnetic-activated cell sorting (MACS) buffer [1% BSA (Sigma) in MACS rinsing solution (Miltenyi Biotech cat. no. 130-091-222)] – this was the 'before-sort' population of cells. Superparamagnetic microbeads (35 μl) conjugated to a CD56 primary antibody

(Miltenyi Biotech, 130-050-401) were mixed into the cell solution and left to incubate for 15 minutes at 4℃. After incubation, the cell solution was diluted with MACS buffer and centrifuged at 657 ***g*** for 6 minutes to collect cells bound to antibody-conjugated beads. The pelleted cells were resuspended in MACS buffer, centrifuged again and finally resuspended in 1 ml of MACS buffer. The cell solution was then passed through a pre-separation filter (Miltenyi Biotech, 130-041-407) and dripped into the column (large cell column; Miltenyi Biotech, 130-042-202) previously equilibrated with 500 µl of MACS buffer and held in a MiniMACS separation unit and MACS multistand (Miltenyi Biotech). Non-retained cells passing straight through the column were collected – these were the 'sort-1 $CD56^-$ cells'. Magnetically retained cells labelled by the anti-CD56 beads were then collected by flushing the column with MACS buffer after it was removed from the magnetic field, releasing the 'sort-1 $CD56^+$' cell fraction. This sort-1 $CD56^+$ population was then passed through a fresh equilibrated column, producing a non-retained 'sort-2 $CD56^-$ fraction' and retained $CD56^+$ cells, which were then released from the column as described above to give the sort-2 $CD56^+$ population. Cell suspensions from each stage of the sorting process were plated at a density of 2000 cells per well into 96-well plates or at equivalent densities on to collagen-coated (0.3 mg/ml; Sigma, C8919) glass coverslips in 24-well plates.

FA treatment

Cells from the $CD56^+$ and $CD56^-$ fractions were exposed for 72 hours to 300 µM oleic acid and 300 µM palmitic acid complexed to BSA at 15 mg/ml in proliferation medium. This resulted in molar ratios of 1.3:1.3:1 oleic acid:palmitic acid:BSA, which is within the range recommended for cell culture studies mimicking serum values (Watt et al., 2012).

Adipogenic induction medium

Cells were exposed to a standard protocol for adipocyte induction from preadipocytes which involves an initial treatment with an 'adipocyte differentiation medium' containing serum (Green and Meuth, 1974), insulin (Green and Kehinde, 1975), dexamethasone (Rubin et al., 1978; Student et al., 1980), 1-methyl-3-isobutylxanthine (a cAMP-phosphodiesterase inhibitor, also known as IBMX) (Russell and Ho, 1976) and PPARγ agonists (De Coppi et al., 2006) for 3 days (Hauner et al., 1989; Tang and Lane, 2012). This was followed by an 'adipocyte nutrition medium' with only a small amount of serum (2%) and insulin for 7 or 15 days (Tchkonia et al., 2006). We refer to these media as 'adipocyte inducing medium' (AIM).

Snap freezing of human skeletal muscle for immunostaining

To characterise CD56 and TE-7 antibody reactivity against human skeletal muscle tissue, biopsies obtained from a young healthy (age 30) and elderly inactive subject (age 83) were snap frozen in dry-ice-cooled isopentane and cryosectioned at 10 µm thickness. Tissue sections were cut and stained as below.

Lipid staining

The lipid (i.e. neutral fat) content of cells was revealed by staining with Oil Red O (Ramírez-Zacarías et al., 1992; Koopman et al., 2001), after all other staining was complete.

Immunostaining

Cryosections and cultured cells were fixed by the addition of 4% paraformaldehyde in ice-cold PBS for 10 minutes. To immunostain cell-surface antigens, cells were blocked for 1 hour with 1% BSA in PBS and then probed with the relevant primary antibodies (supplementary material Table S2). For intracellular antigens, cells were permeabilised after fixation by addition of 0.2% Triton X-100 in PBS with 1% BSA and NaN_3 (0.01%) for 10 minutes, then blocked and incubated with primary antibodies. Unlabelled primary antibody binding was followed by incubation with species-specific fluorescently labelled secondary antibodies (supplementary material Table S3) and 10 minute incubation in Hoechst 33342 (Sigma) dye before mounting in Dako 'anti-fade gold' medium (Dako UK Ltd, Cambridgeshire, UK). Stained cryosections were mounted in Dako 'anti-fade gold' with DAPI (Dako).

Image capture and analysis

Immunofluorescent probes were illuminated by epifluorescence delivered by liquid light guides and signals were visualised through red (filter set 45 HQ Texas red shift free), green (filter set 44 FITC special shift free) and blue (filter set 49 DAPI shift free) band-pass filters (Carl Zeiss, Cambridge, UK) on an AxioPlan microscope (Carl Zeiss; 10×, 20×, 40× objectives with 0.25, 0.75, 0.95 numerical apertures, respectively). To establish and correct for the 'dark current' of the camera's sensor a 'black reference' was performed prior to image acquisition and again during each session as necessary using Axiovision software (version 4.3.2 Carl Zeiss Microimaging). To avoid pixel saturation the optimal exposure was checked using the 'Overexposure' feature. Grey-scale images were captured using an AxioCam MRm digital camera (Zeiss) and Axiovision software was used to assign pseudo red, green or blue colour tones to micrographs to correspond with the emission wavelength of the secondary antibody.

To ensure strict comparability, treated and control cells from the same experiment maintained for the same time in culture, and stained with the same label of interest, were photographed at identical exposures and in the same microscopy session. Image analysis was performed as previously described by Agley et al. (Agley et al., 2012) with some modifications. Local background intensity values were subtracted from measurements to account for potential field to field variation in non-specific background fluorescence (Waters, 2009). In the micrographs used for figures, colour balance was altered slightly and an unsharp mask was applied (amount: 150–200, radius: 2). All post-acquisition formatting was in accordance with suggested guidelines for digital images (Rossner and Yamada, 2004). Only raw, unadjusted 8 or 16 bit monochrome images were used for quantitative analysis.

Cell counting for marker expression

Normally, three replicate wells were plated and analysed per condition for all experiments; if the number differed, it is marked either on the figure or in its legend. Cells from the sorted fractions were plated in 96-well plates and after immunostaining, five to six images of non-overlapping fields were captured at standardised exposures. At least 1000 cells were counted per well from five to six non-overlapping fields of view in replicate wells using the 'Count' feature of Photoshop CS5 Extended software (Adobe Systems Inc.).

Measurement of cellular lipid content

To assess changes in cellular lipid content in the $CD56^+$ and $CD56^-$ cell populations, a specific colour range selection mask (Agley et al., 2012) was created to select a threshold for Oil-Red-O^+ lipid droplets. This mask could be applied equally to all fields of view and sensitively discriminated even very small lipid droplets to give unbiased measurements that were exactly reproducible. The area of Oil-Red-O staining in each field of view was measured and normalised to nuclear number to give average lipid content per cell. Desmin or TE-7 staining confirmed the myogenic or fibroblast identity of the cultures that were analysed.

Quantification of nuclear transcription factor expression

To measure the expression of myogenic and adipogenic transcription factors in individual nuclei, a representative colour range selection mask for blue Hoechst 33342 (Sigma) stained nuclei was created and applied to select all nuclei in a field of view. This live selection was then transferred to the layer corresponding to antibody staining for PPARγ or C/EBPα, so that the fluorescent signal (raw non-averaged grey-scale) could be objectively quantified on a cell-by-cell basis, revealing the full range of transcription factor expression in a large population of cells. In all cases, measurements were made on all nuclei from at least 15 fields of view (per cell type and condition) sampled from three independent experiments performed on cells derived from a representative biopsy.

Statistics

Unpaired *t*-tests or Mann–Whitney U-tests (when data were non-normally distributed) were used to compare differences between groups. For analysis of more than two groups, significance was assessed by two-way mixed model analysis of variance (ANOVA) with post-hoc Bonferroni adjustment. In all cases n numbers are given for experiments and an alpha value of $P<0.05$ was accepted as statistically significant.

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Author contributions

C.A. designed the experiments, undertook all experimental work, analysed the data and wrote the manuscript. A.R., C.V. and N.L. were involved in designing the experiments and writing the manuscript. S.H. took the muscle biopsies, designed the experiments, and wrote the manuscript. All authors read and approved the final manuscript.

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