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Fat pad-derived mesenchymal stem cells as a potential source for cell-based adipose tissue repair strategies

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Abstract

Background: Mesenchymal stem cells are able to undergo adipogenic differentiation and present a possible alternative cell source for regeneration and replacement of adipose tissue. The human infrapatellar fat pad is a promising source of mesenchymal stem cells with many source advantages over from bone marrow. It is important to determine whether a potential mesenchymal stem-cell exhibits tri-lineage differentiation potential and is able to maintain its proliferation potential and cell-surface characterization on expansion in tissue culture. We have previously shown that mesenchymal stem cells derived from the fat pad can undergo chondrogenic and osteogenic differentiation, and we characterized these cells at early passage. In the study described here, proliferation potential and characterization of fat pad-derived mesenchymal stem cells were assessed at higher passages, and cells were allowed to undergo adipogenic differentiation.

Materials and methods: Infrapatellar fat pad tissue was obtained from six patients undergoing total knee replacement. Cells isolated were expanded to passage 18 and proliferation rates were measured. Passage 10 and 18 cells were characterized for cell-surface epitopes using a range of markers. Passage 2 cells were allowed to undergo differentiation in adipogenic medium.

Results: The cells maintained their population doubling rates up to passage 18. Cells at passage 10 and passage 18 had cell-surface epitope expression similar to other mesenchymal stem cells previously described. By staining it was revealed that they highly expressed CD13, CD29, CD44, CD90 and CD105, and did not express CD34 or CD56, they were also negative for LNGFR and STRO1. 3G5 positive cells were noted in cells from both passages. These fat pad-derived cells had adipogenic differentiation when assessed using gene expression for peroxisome proliferator-activated receptor $\gamma 2$ and lipoprotein lipase, and oil red O staining.

Discussion: These results indicate that the cells maintained their proliferation rate, and continued expressing mesenchymal stem-cell markers and pericyte marker 3G5 at late passages. These results also show that the cells were capable of adipogenic differentiation and thus could be a promising source for regeneration and replacement of adipose tissue in reconstructive surgery.

Introduction

There is ever-increasing clinical need for regeneration and replacement of adipose tissue, in reconstructive surgery to replace soft tissue lost due to trauma, disease and in congenital anomalies, as well as in cosmetic surgery (1,2).

Use of synthetic materials for example collagen, to enhance or improve soft tissue contours, invariably results in foreign body reaction (3). A patient's own tissue is preferable to allografts and xenografts to avoid potential complications, such as pathogen transmission and immune rejection. Autologous free adipose tissue grafts have been used in many clinical settings to restore defects such as in breast reconstruction, but they have inconsistent vascularization, and result in unpredictable shrinkage up to 40–60%, and thus, poor cosmesis (4). These procedures are also associated with donor site morbidity and deformity

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(5), and frequently additional procedures might be necessary for tissue harvest. Adipose cell suspensions obtained through liposuction result in significant mechanical trauma to adipocytes (6), mature adipocytes obtained do not have potential to proliferate and have poor ability for volume retention when used for tissue reconstruction (1). Cell-based repair strategies using preadipocytes have been explored, but these cells are difficult to isolate, show unpredictable variability based on age and anatomical site of origin, and exhibit limited proliferation (7–9).

A potential alternative to current treatment modalities is use of tissue engineering applications with mesenchymal stem cells (MSCs) that have been identified in many tissues, including bone marrow, adipose tissue and the pad (10–13). These cells have excellent proliferative potential and have been shown to undergo adipogenic differentiation under defined culture conditions (14). A tissue engineered graft of a patient's own MSCs would overcome shortcomings of current treatment modalities.

Adipose tissue engineering approaches have been described using bone marrow-derived MSCs (9,15), but initial harvest of bone marrow can be painful and has associated morbidities including risk of wound infection and sepsis (14). In addition, these cells form only 0.001–0.01% total nucleate cells in bone marrow aspirates (16). With a person's increasing age, there is reduction in bone marrow cellularity including of MSCs (17,18). Some studies have also shown age-related decline in osteogenic potential of these cells (19,20). The fat pad, however, is easily accessible with less discomfort to the patient and has a greater yield of MSCs than bone marrow (21). Fat pad-derived MSCs also do not seem to have any age-related decline in proliferative and differentiation potential (22). Although a biomechanical cadaveric study (23) indicated that the infrapatellar fat pad may have a biomechanical function, and may play a role in anterior knee pain syndrome, there was no clinical evidence that resecting the fat pad would cause problems. The infrapatellar fat pad is commonly resected at arthroscopy for improved surgical visualization, and in arthroplasty to prevent possible impingement of fat by the prosthesis. No adverse long-term side effects have been noted following resection of the infrapatellar fat pad (24).

It is important to determine whether a potential mesenchymal stem-cell source exhibits tri-lineage differentiation potential, and is able to maintain its proliferation potential and cell-surface characterization on expansion in culture. We have previously shown that MSCs derived from the fat pad can undergo chondrogenic and osteogenic differentiation, and we characterized these cells at early passage (22,25). In this study, proliferation potential and characterization for fat pad-derived MSCs were assessed at higher passages, and cells were allowed to undergo adipogenic differentiation in appropriate culture medium.

Materials and methods

Infrapatellar fat pad tissue was obtained from six patients undergoing total knee replacement for osteoarthritis, following ethical committee approval and written informed consent. Five millilitres of fat pad weighting between 4 and 5 g was dissected from each knee and cells were isolated by digestion with 0.2% (vol/vol) collagenase I (Invitrogen, Paisley, UK) for 3 h at 37 °C with constant agitation. Released cells were sieved (70-μm mesh) and washed in basic medium consisting of Dulbecco's modified Eagle's medium supplemented with 20% (vol/vol) foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Cambrex, Wokingham, UK), with L-glutamine (2 mM). Stromal cells were separated from floating adipocytes by centrifugation at 300 ***g*** for 5 min, and were counted and plated at 100 000 cells/cm^2 in monolayer culture in basic medium. Five millilitres of tissue yielded around 7.5 million mononuclear cells. Cultures were maintained at 37 °C with 5% CO_2 and normal oxygen (20%).

Calculation of cell population doublings per day and proliferation rates

Growth kinetics of cell populations for different passages were calculated using an equation from a previously described method (26):

$$\text{Population doublings per day} = (\ln (N/N^{o}))/t$$

Where N is total number of cells at the end of the time period, N^{o} is total number of cells at the beginning of the time period; t is time period in days. Cell number was determined by counting using a cytometer. Viability of cells was determined by staining with trypan blue, then assessing cells able to exclude the dye.

Cell-surface epitope characterization and flow cytometry

Confluent passage 10 and passage 18 cells were stained using a panel of antibodies for cell-surface epitopes previously used for passage 2 cells (27). This included antibodies to CD13 (aminopeptidase N), CD44 (hyaluronan receptor), CD90 (Thy-1), LNGFR (low affinity nerve growth factor receptor), STRO-1 (marker for bone marrow-derived stem cell) and CD56 (neural cell adhesion molecule, NCAM) from BD Biosciences, Oxford, UK; CD29 (β1 integrin), CD105 (SH2 or endoglin) and CD34 (marker for haematopoetic cells) from Dako, Ely, UK; and 3G5 (marker for vascular pericytes) courtesy of

Dr Ann Canfield, University of Manchester, UK. Cells were incubated for 1 hour with mouse primary antibodies (neat 3G5 and 1:100 dilution for others) followed by FITC-conjugated anti-mouse IgM secondary antibody (1:40 dilution) (Dako). For controls, nonspecific monoclonal mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was substituted for the primary antibody. Cells were incubated in 1:100, 4′,6-diamidino-2-phenylindole stain for 5 min and images were captured using an Axioplan 2 microscope with Axiocam HRc camera and AxioVision 4.3 software (all from Carl Zeiss Ltd, Welwyn Garden City, UK).

Cells were also analysed using flow cytometry. Cells in monolayer were detached using trypsin (0.05% with 5 mM EDTA), washed and incubated in mouse primary antibodies (neat 3G5 and 1:100 dilution for others) followed by FITC-conjugated anti-mouse IgM secondary antibody (1:40 dilution). They were then re-washed, suspended at 1 million cells/ml and assayed in a flow cytometer (Dako cytomation cyan).

Adipogenic differentiation culture

For adipogenic differentiation, passage 2 cells were seeded at 5000 cells/cm^2 density in six-well plates in basic medium. Medium was changed every 3 days until the cells were confluent. Cells were then cultured in adipogenic inducing medium consisting of basic medium with 10 μg/ml insulin, 1 μM dexamethasone, 100 μM indomethacin and 500 μM 3-isobutyl-1-methyl xanthine (IBMX) (all from Sigma, Poole, UK), for 72 h. This was followed by adipogenic maintenance medium consisting of basic medium with 10 μg/ml insulin for 24 h. The cycle was repeated four times over 16 days and cells were then cultured in adipogenic maintenance medium for one further week with medium changed once in the week (11,14,28). Parallel cell lines were cultured in control basic medium without adipogenic differentiation and maintenance factors.

Gene expression analysis

Three wells were used for extraction of RNA examine gene expression of peroxisome proliferator-activated receptor γ2 (PPARγ2) gene and that of lipoprotein lipase (LPL). Quantitative real-time gene expression analysis was performed. Total RNA was extracted using Tri Reagent (Sigma) from passage 2 cells in monolayer; cDNA was generated from 10 to 100 ng total RNA by using reverse transcription followed by poly(A) polymerase chain reaction (PCR) global amplification (29). Globally amplified cDNAs were diluted 1:1000 and 1 μl aliquot of diluted cDNA was amplified by quantitative real-time PCR in final reaction volume of 25 μl by using MJ Research Opticon with an SYBR Green Core Kit (Eugentec, Seraing, Belgium). Gene-specific primers were designed within 300 base pairs 3′ region of the relevant gene with use of ABI Primer Express software (Applied Biosystems, Foster City, CA, USA). Gene expression analyses were performed relative to β-actin and calculated using the $2^{-\Delta\Delta Ct}$ method (30). All primers (Invitrogen) were based on human sequences: β-actin, 5′-AAGCCACCCCACTTCTCTC-TAA-3′ (forward) and 5′-AATGCTATCACCTCCCC TGTGT-3′ (reverse); PPARγ2, 5′-TCAGGTTTGGGCGG ATGC-3′ (forward) and 5′-TCAGCGGGAAGGACTT TATGTATG-3′ (reverse); LPL, 5′-GAACCGCTGCAA CAATCTGGGCTATGA-3′ (forward) and 5′-TGCTG CTTCTTTTGGCTCTGACTTTATTGA-3′ (reverse).

Oil red O staining

Stock solution of 0.5% (w/v) oil red O (0.7 g in 200 ml of isopropanol) was prepared and filtered through a 0.2-μm filter. Working solution was prepared by diluting 6 ml stock solution in 4 ml distilled water; working solution was kept for 1 h at room temperature before being filtered once more. Cells of the remaining three wells per six-well plate, were fixed in 10% solution formaldehyde in Delbecco's phosphate-buffered solution (Cambrex, Wokingham, UK) for 1 h at 40 °C, washed in 60% isopropanol, then stained in 1 ml oil red O solution (in 60% isopropanol) for 10 min, followed by five rinses in distilled water. Dye retained by cells was eluted by incubation in 100% isopropanol for 15 min.

Statistical analyses

Experiments were performed separately with cells from six patients and all experiments were in triplicate. Cell expansion and gene expression data are presented as mean and standard deviation. One-way analysis of variance (ANOVA) with Bonferroni's correction was carried out to compare population doublings over the 18 passages. Student's paired *t*-test was used to analyse results from gene expression studies and to determine levels of significance. Statistical analyses were conducted with SPSS statistical software (version 11.5) (SPSS Inc., Chicago, IL, USA) and significance was set at P-value <0.05.

Results

No effect of passage number on cell population doublings

Cells from fat pad samples were expanded to passage 18 to determine whether rate of population doublings was affected by progressive passaging. Cells from sequential passages replated at 1:3 dilution of confluence, took

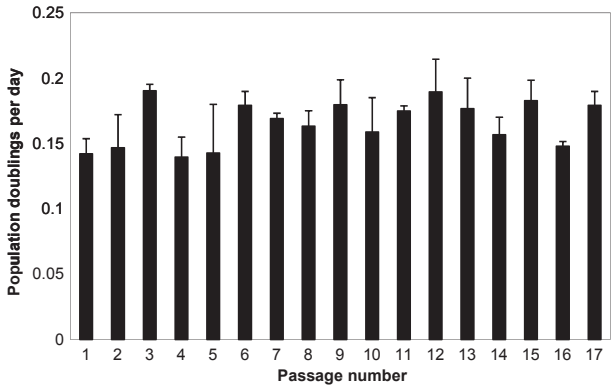

Figure 1. Cell population doublings per day for fat pad-derived cells from different passages, expanding in monolayer culture, in basic medium. Data are mean ± standard deviation.

7–10 days to regain confluence level. There was no significant difference between population doubling per day for any of the 18 passages (Fig. 1).

Cell-surface staining and flow cytometry indicate maintenance of cell-surface epitope profile in passage 10 and passage 18 cells

Cell-surface epitope profile characterization of passage 10 and 18 fat pad-derived MSCs was performed using the following panel of antibodies. Cells from both passages stained strongly for CD13, CD44, CD90 and CD105 (markers of MSCs), and for CD29 (marker of β1 integrin) (Fig. 2). Cells stained poorly for LNGFR and STRO1 (markers of bone marrow-derived MSCs), CD34 (marker of haematopoetic lineage) and CD56 (marker of NCAM). Occasional cells stained positively for 3G5 (pericyte marker). No staining was observed on IgG control cells.

There was similar increase in fluorescence for CD13, CD29, CD44, CD90 and CD105 of cells from both passages, using flow cytometry. There was no detectable increase in fluorescence when cells were labelled with antibodies to 3G5, LNGFR, STRO1, CD34 and CD56. The fluorescence histogram for the sample containing no primary antibody had a little expected difference compared to IgG control cells, due to non-specific binding. The fluorescence histogram of no primary control cells identified distinct peaks over a wide fluorescence range.

Fat pad-derived cells cultured in adipogenic medium had increased expression of PPARγ2 and LPL, and increased lipid vacuole accumulation displayed by oil red O staining

Cells cultured under adipogenic conditions had 391-fold increase in expression of PPARγ2 and more than 2 million-fold increase in expression of LPL ($P < 0.05$) (Fig. 3).

Expression of adipocyte-specific genes during adipocyte differentiation was associated with morphological changes. Vacuoles were observed in confluent fat pad-derived cells after 3 days in adipogenic culture. Then there was gradual increase in number and size of cells and of vacuoles. By day 16, many cells had vacuoles occupying most of the cytoplasm, but no significant morphological change was observed during final adipogenic maintenance medium culture for 1 week. Oil red O staining was performed to confirm nature of observed vacuoles and confirmed that they were lipid, as shown in Fig. 4. No such vacuoles were observed in cells cultured in control medium and no staining was seen in these cells.

Discussion

Ex vivo expanded MSCs may need to undergo a large number of cell divisions in monolayer culture to reach a number sufficient for clinical application. This may lead to cell senescence, and it is important to determine whether characterization is altered by passage, in monolayer culture. Full characterization of fat pad-derived MSCs at late passage is important to achieve greater understanding of their origin and their repair potential. Cell-surface epitope profile of synovial tissue-derived MSCs has been shown to be stable during expansion up to passage 10 (31). Although some previous studies have looked at cell-surface characterization of fat pad-derived cells at early passage (12,22,25,32), no previous study has observed them at late passage. To investigate effects of passage on expression of cell-surface epitopes within the fat pad population, cell-surface staining and flow cytometry were performed on passage 10 and passage 18 cells, employing a panel of antibodies previously used for passage 2 cells.

Passage 10 and passage 18 cells were consistently expressed CD13, CD29, CD44, CD90 and CD105, and did not express LNGFR, STRO1, CD34 or CD56, suggesting a homogenous population at this stage. Cell-surface epitope characterization suggested that fat pad cell population had surface expression characteristics of MSCs (14,33–35), and these were consistently maintained over passage. Fat pad-derived MSCs have previously been shown to express CD29, CD90 and CD105 (36). Although we have not found fat pad-derived MSCs able to express LNGFR at lower passage numbers previously and higher passages in this study, English *et al.* (37) found LNGFR expression of 31 ± 17% in early passage at ∼11 population doublings. Lack of CD34 expression in fat pad-derived cells suggests that there was no contamination with haematopoetic cells during their isolation.

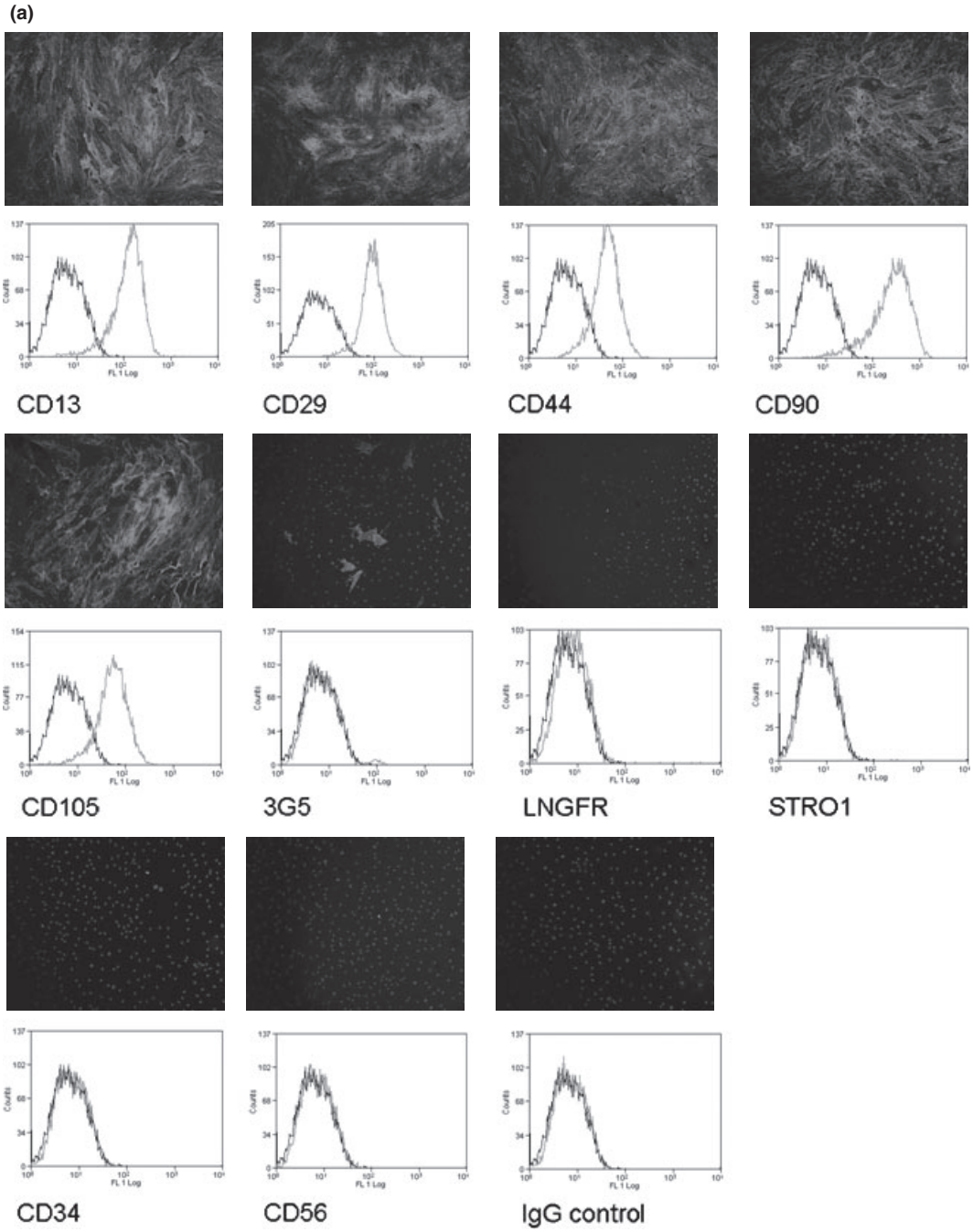

Figure 2. Cell-surface epitope characterization of passage 2 (a) (21), passage 10 (b) and passage 18 (c) fat pad-derived MSCs using a panel of antibodies. Cell-surface staining using FITC conjugated secondary antibody (green) and DAPI (blue) shows that cells stained strongly for CD13, CD29, CD44, CD90 and CD105, and poorly for LNGFR, STRO1, CD34 and CD56. Occasional cells stained for 3G5. No staining was observed for on IgG controls. The staining pattern was confirmed by flow cytometry, and shows increase in fluorescence (green) compared to autofluorescence (black) for CD13, CD29, CD44, CD90 and CD105.

Anti-CD56 antibody recognizes neural (38,39) and myogenic stem cells (40,41), and its absence in the expression profile of fat pad-derived cells further confirms lack of contamination by cells of other tissues during isolation and expansion. The consistent pattern of expression demonstrates that adherence to plastic, and selection through proliferation and passaging allowed selection of a homogenous MSC population.

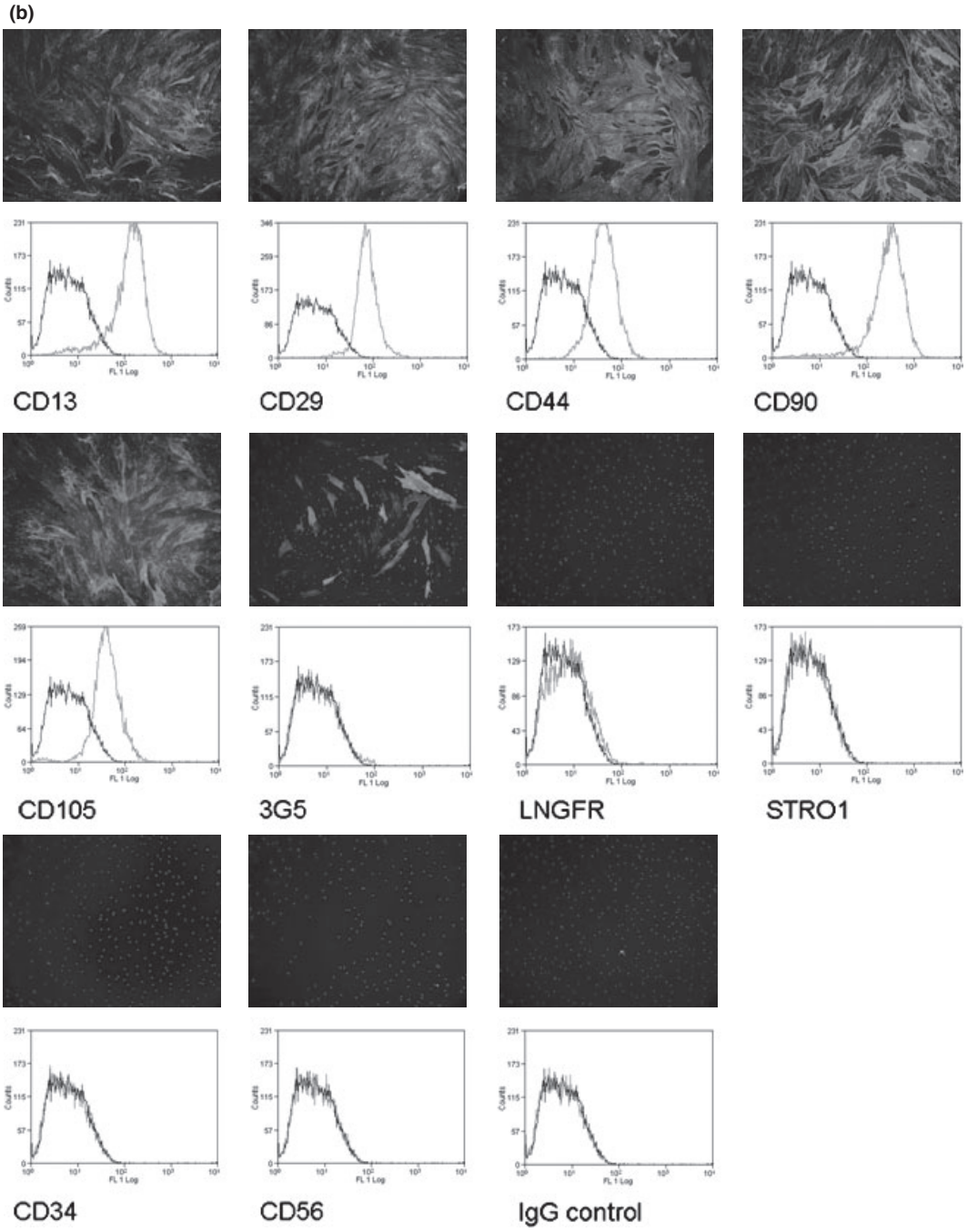

Figure 2. (Continued)

Our gene expression results showed that the fat pad-derived cells had the potential to undergo adipogenic differentiation when cultured in adipogenic medium. Previous studies on adipogenesis on fat pad-derived MSCs have either looked only at staining (37) or failed to perform quantitative gene expression studies (32). Ours, thus, is the first one to observe both these parameters. Morphological changes during adipocyte differentiation were associated with expression of adipocyte-specific genes. PPARγ2 is a central regulator of fat cell differentiation and its activation is a critical step in adipogenesis (41,42); PPARγ2 is a key transcription factor of adipogensis *in vitro* and *in vivo* (43, 44). LPL is involved in lipid transport, storage and metabolism. Both

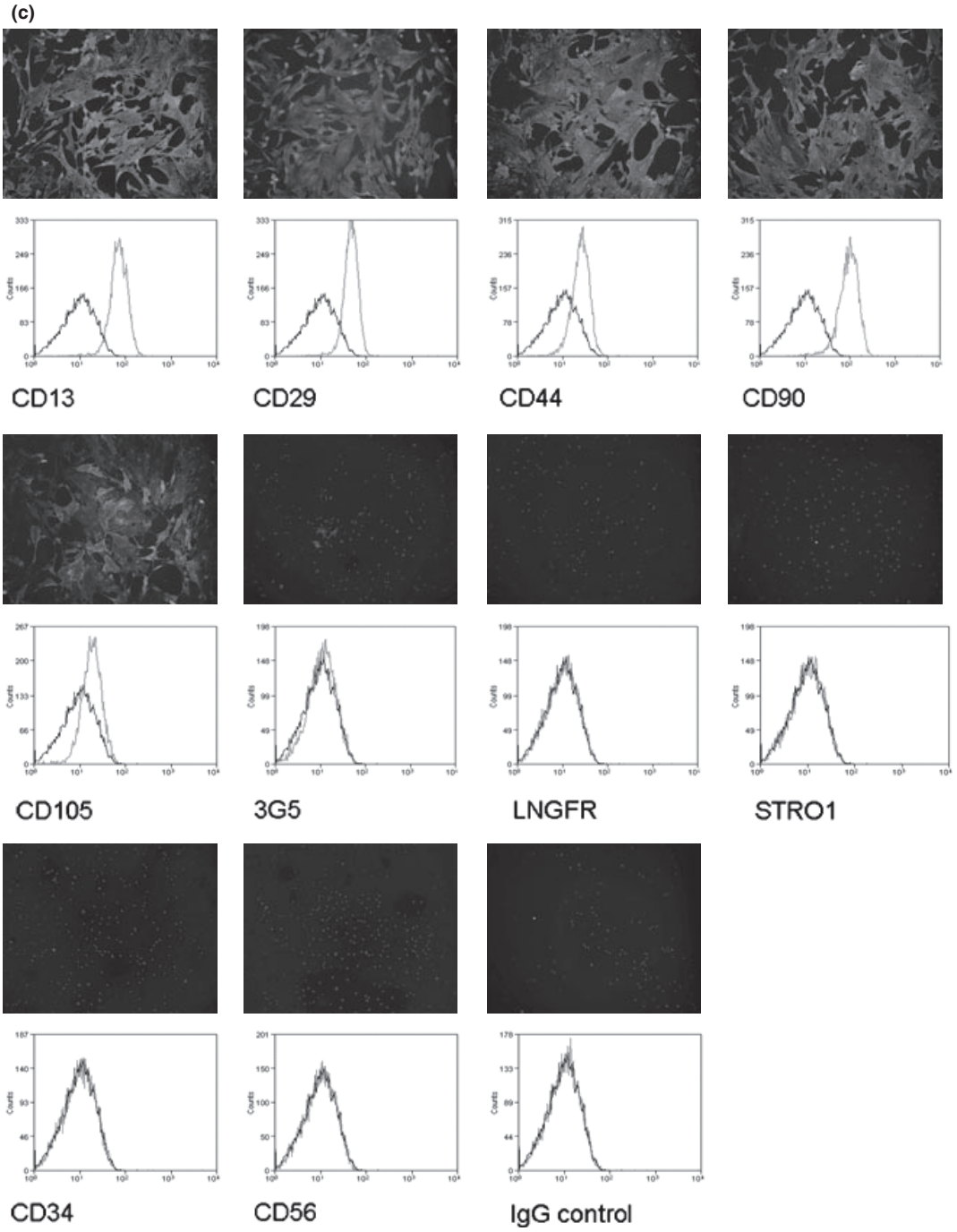

Figure 2. (Continued)

PPARγ2 and LPL are early adipocyte markers (45). Cells cultured under adipogenic conditions had significantly greater expression of PPARγ2 and LPL. Gene expression studies were performed relative to beta-actin rather than to glycerol-3-phosphate dehydrogenase (GAPDH); GAPDH is a key enzyme in biosynthesis of triglycerides and a late marker of adipogenic differentiation, making it unsuitable as a reference gene.

In fat pad-derived cells cultured under adipogenic differentiation culture conditions, size and number of lipid vacuoles increased with time, suggesting greater degrees of maturation (9). By day 16, most cells had lipid vacuoles

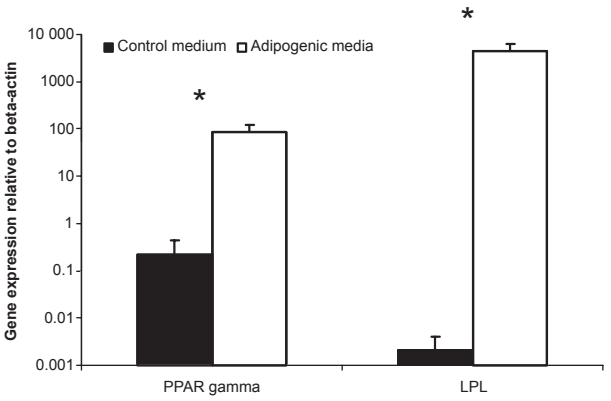

Figure 3. PPARγ2 and LPL relative gene expression levels are significantly greater for fat pad-derived MSCs cultured in adipogenic medium than for cells cultured in control medium. Data are mean ± standard deviation. *$P < 0.05$ (Student's paired *t*-test).

occupying most of the cytoplasm, probably the result of undergoing terminal differentiation (46). Oil red O staining revealed triglyceride accumulation within the cells exhibiting typical adipogenic morphology. Cells cultured in control medium failed to express genes suggestive of adipogenic differentiation and showed no staining of lipid vacuoles on oil red O staining.

To induce adipogenic differentiation, cells were cultured in adipogenic medium containing insulin, dexamethasone, indomethacin and IBMX. Insulin, dexamethasone and IBMX have been shown to be sufficient to stimulate adipogenic differentiation (11,14,45). Insulin is needed to generate the substrate glycerol 3-phosphate, which is needed for biosynthesis of triglycerides (47); also, it is known to promote proliferation and differentiation of preadipocytes (7). High concentrations of insulin mimic the role of insulin-like growth factor (IGF-1) (48) and have mitogenic effects. The anti-inflammatory drug indomethacin is a PPARγ2 activator and was also used in our medium as it is a strong inducer of adipogenesis (49).

Adipogenesis of undifferentiated MSCs is made up of two stages: determination of adipocyte lineage, and adipogenic differentiation. Molecular pathways involved in the second stage have been studied extensively (50), but those of the first stage are not well understood (51). Our results show that serum deprivation is not necessary for adipogenic differentiation as has previously been suggested (52,53), and supporting previous observations (54). Our results support the previous finding that adipogenic differentiation of MSCs is only possible when cells are confluent (55). Our results also support previous finding that MSCs require several cycles of hormonal stimulation to exit from the cell cycle, a feature necessary to achieve commitment to the adipogenic lineage (51).

In a patient-matched quantitative comparison, MSCs from adipose synovium have been shown to demonstrate higher proliferative potential and colony-forming efficiency compared to subcutaneous fat-derived cells, both in mixed-population and in single-cell-derived cultures. In addition, adipose synovium MSCs also have been shown

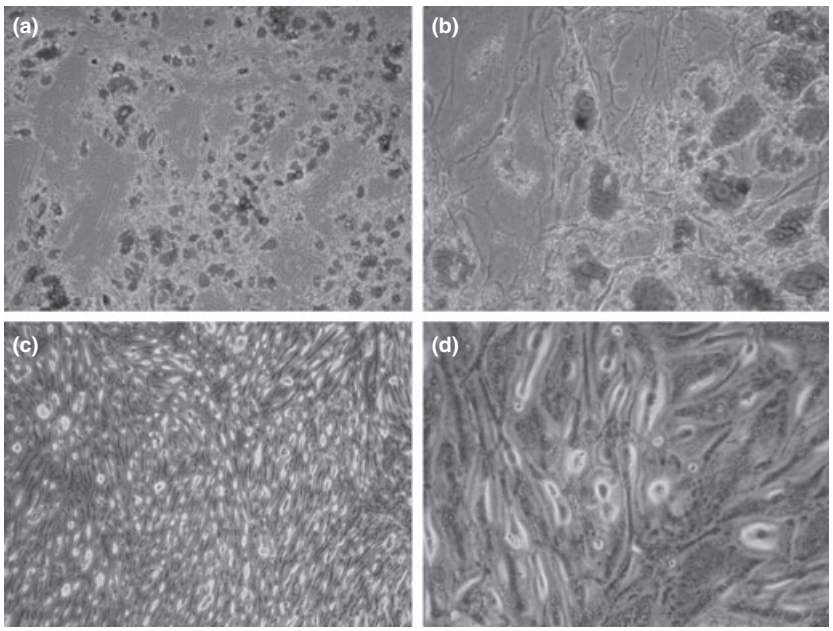

Figure 4. Oil red O staining for fat pad-derived MSCs cultured in adipogenic medium at ×10 (a) and ×40 (b) magnification show significantly greater staining than cells cultured in control medium at ×10 (c) and ×40 (d) magnification.

to exhibited greater chondrogenic and osteogenic potential compared to subcutaneous fat MSCs (56). Cell expansion is an important consideration in any potential MSC-based treatment, and fat pad-derived cells have previously been shown to have better proliferation potential and tri-lineage differentiation potential compared to adipose tissue, and in this study, we have shown that they also maintain their proliferation rate and cell-surface characterization in late passages. Cell-based adipose tissue repair strategies using fat pad-derived MSCs will not have disadvantages of exhibiting limited proliferation as shown by preadipocytes. Harvesting MSCs from all tissue sources is associated with potential risks, and benefits of using MSCs from the fat pad need to be balanced against the small potential risk of joint sepsis. A comparative patient-matched study is planned to determine effects of passage on proliferation and cell-surface characterization, on MSCs from different sources. The next challenge would be to establish how these cells behave *in vivo*. To date, there have been no *in vivo* studies on fat pad-derived MSC constructs undergoing adipogenesis, and it remains to be seen whether adipogenic potential is maintained *in vivo*.

Conclusion

Our results show that fat pad-derived MSCs exhibited tri-lineage differentiation potential and maintained their proliferation potential while continuing to express MSC markers and pericyte marker 3G5, at late passage. We believe that these cells are a promising source for regeneration and replacement of adipose tissue for reconstructive surgery.

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