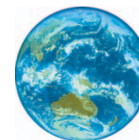
CLINICAL REVIEW



ANZJSurg.com

Human adipose-derived stem cells: isolation, characterization and applications in surgery

Michelle Locke,* John Windsor† and P. Rod Dunbar‡

*Dunbar Laboratory and
‡Maurice Wilkins Centre for Molecular Biodiscovery, School of Biological Sciences, and
†Department of Surgery, University of Auckland, Auckland, New Zealand

Key words
adipose-derived stem cells, adipose tissue, differentiation, isolation, mesenchymal stem cells.

Abbreviations
ASC, adipose-derived stem cell; BMSC, bone marrow-derived stem cell; CD, cluster of differentiation; HIV, human immune virus; HSV, Herpes simplex virus; Hve, herpesvirus entry mediated; IGF, insulin-like growth factor; i.p., intraperitoneal; i.v., intravenous; IL, interleukin; SVF, stromal vascular fraction; T3, triiodothyronine; TGF, transforming growth factor; UCB, umbilical cord blood; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.

Correspondence
Dr Michelle Locke, Dunbar Laboratory, School of Biological Sciences, Thomas Building, University of Auckland, 3a Symonds Street, Auckland, New Zealand.
Email: michellebjoyce@yahoo.co.nz

M. Locke MBChB; **J. Windsor** BSc, MBChB, MD, FRACS; **P. R. Dunbar** MBChB, PhD.

Accepted for publication 12 July 2008.

doi: 10.1111/j.1445-2197.2009.04852.x

Abstract

The ideal stem cell for use in functional tissue engineering needs to be abundantly available, harvested with minimal morbidity, differentiated reliably down various pathways and able to be transplanted safely and efficaciously. Adult human adipose tissue contains a population of mesenchymal stem cells, termed 'adipose-derived stem cells' (ASC), which seem to fulfil most, if not all, of these criteria. ASC can be harvested readily, safely, and in relative abundance by modern liposuction techniques. They are capable of differentiating into other mesenchymal tissue types, including adipocytes, chondrocytes, myocytes and osteoblasts. They also show angiogenic properties, with recent evidence of a potential role in healing radiotherapy-damaged tissue, possibly due to their secretion of vascular endothelial growth factor. Similarly, they may have a role in healing chronic wounds, and as such are being investigated in phase 1 trials for their ability to aid healing of recurrent Crohn's fistulae. Subsequently they have a wide range of potential clinical uses in all fields of surgery. This article reviews the current and potential clinical applications of ASC in relation to surgery, as well as methods for their isolation, differentiation and molecular characterization.

Introduction

Stem cells exist in an undifferentiated state, and exhibit both the capacity to self-renew, and the capability to differentiate into more than one type of cell. Although embryonic stem cells seem to exhibit unlimited differentiation potential both *in vitro* and *in vivo*, they are subject to significant ethical, legal and political concerns and are not generally available in current medical practice or research. Stem cells from adult tissue, on the other hand, suffer from few such restrictions.

Multipotent stem cells can be isolated from various mesenchymal tissue sources in adults, most commonly bone marrow. The harvest of bone marrow stem cells (BMSC) has practical constraints. These include pain at the harvest site, and harvest of only a small volume of bone marrow (and therefore a small number of stem cells),[1] meaning that they are likely to require *ex vivo* expansion of the cells to obtain clinically significant cell numbers. This is especially true for the elderly or patients with malignancy,[2] who are most likely to have low BMSC numbers. Umbilical cord blood (UCB) from the placenta

© 2009 The Authors
Journal compilation © 2009 Royal Australasian College of Surgeons

of term infants also contains mesenchymal stem cells. The use of UCB has been limited by the practical difficulties in obtaining and isolating the mesenchymal stem cells at the time of birth and ensuring adequate long-term storage for autologous use, and the fact that their differentiation potential seems to be lower than that of BMSC.[3]

Early adipose stem cell research began in 1992,[4] when investigators used porcine preperitoneal fat, which they reported has similar morphology to human subcutaneous adipose cells, to culture the stromal vascular fraction (SVF) of cells in media with heparin and endothelial cell growth factor. This produced cells that stained positively for von Willebrand factor (vWF), α-smooth muscle cell actin and cytokeratin. They were termed 'microvascular endothelial cells'. But the SVF has, more recently, been shown to include multipotent mesenchymal stem cells,[1,5,6] which may reside in the perivascular region of the stroma.[7]

Adipose tissue is derived from embryonic mesoderm. As mesenchymal stem cells, the isolated cells are capable of forming bone, cartilage and muscle, as well as fat.[1,5,6,8–12] These stem cells have been variously termed pre-adipocytes, stromal cells, processed lipoaspirate cells, multipotent adipose-derived stem cells, and adipose-derived adult stem cells. A recent consensus reached by investigators at the 2004 conference of the International Fat Applied Technology Society has settled on the term 'adipose-derived stem cells' (ASC).[13]

Abundant numbers of ASC can be derived from lipoaspirate, the waste product of liposuction surgery. Processing 300 mL of lipoaspirate routinely yields between 1×10^7 and 6×10^8 ASC[1,14–17] with >90% cell viability.[14,15] The yield compares favourably with a bone marrow aspirate.[1,18] Compared with BMSC, ASC are more easily cultured and grow more rapidly.[1,17,19] They can also be cultured for longer than BMSC before becoming senescent.[3] All of these qualities make ASC a useful source of mesenchymal stem cells.

Sources and isolation of adipose derived stem cells

Adipose stem cells can be found in any type of white adipose tissue, including subcutaneous and omental fat. In recent years the prevalence of obesity has been rapidly rising throughout the Western world and, along with this, the number of patients undergoing elective liposuction has also increased significantly. The American Society for Aesthetic Plastic Surgery states that the total number of all cosmetic procedures has increased more than fourfold since 1997, with 455 489 liposuction procedures performed in the USA in 2005.[20] Liposuction is considered a safe and well-tolerated procedure, with a complication rate of around 0.1%.[21–24] Liposuction routinely removes between a few hundred millilitres and several litres of fat. Although the lipoaspirate has been subjected to suction forces during aspiration, research shows that 98–100% of the adipose cells in the lipoaspirate are viable.[19,25] Following the procedure, the lipoaspirate is normally discarded as medical waste. Given the safety and popularity of liposuction, lipoaspirate seems an ideal source of ASC, and a safe autologous cell source for clinical use.

In the 1960s, Rodbell pioneered the technique of isolating adipose cells from rat tissue.[26] Minor modifications allowed this to be successfully applied to human tissue in the 1980s.[27] Lipoaspirate should be processed as soon after harvest as possible, although one author suggests that the lipoaspirate can be kept at room temperature overnight before stem cell isolation if necessary.[15] There are a number of similar methods reported in the literature for isolating the adipose cells from the lipoaspirate.[1,5,14,15] The process is summarized in Figure 1. In brief, the lipoaspirate is extensively washed and the red cells are lysed. The floating adipose tissue is digested with collagenase to break down the extracellular matrix, filtered and centrifuged. After spinning down, the resulting pellet is known as the SVF. In our experience, processing of large volumes of lipoaspirate can be performed efficiently over 2–3 h, with expected yields of approximately 2.4×10^4 cells per mL of fat processed (data not shown). Cells within the SVF subsequently adhere readily to plastic tissue cultureware under standard incubation conditions, and growth of these cells is evident within a few days (Fig. 2).

The SVF contains an unpurified population of stromal cells, which includes the ASC. The other cell types that may be present in SVF are those found in the lipoaspirate, namely endothelial cells, smooth muscle cells, pericytes, fibroblasts, and circulating cell types such as leucocytes, haematopoietic stem cells or endothelial progenitor cells.[28] Many authors use the entire unpurified SVF in their experiments, on the basis that the ASC are adherent to the plastic tissue cultureware, so they are self-selected out of the SVF during subsequent tissue culture passages.[29] As few as one in 30 of the SVF cells adhere to the plastic,[17] and there is progressive loss of haematopoietic lineage cell markers (such as CD11, CD14 and CD45) with successive cultures of ASC.[17] Adherence to plastic tissue cultureware, however, is not a feature that is specific to ASC, because fibroblast cells also behave in this manner. Some critics have suggested that even a low fraction of contaminating cells such as haematopoietic stem cells could be the source of differentiation seen in ASC experiments.[30]

Purification by magnetic bead coupling has been performed by some researchers[31,32] to remove $CD45^+$ cells (leucocytic/haematopoietic lineage) and $CD31^+$ cells (endothelial lineage) from the isolated cells prior to differentiation experiments. Given the relative simplicity of such sorting procedures, it would seem reasonable to advocate that ASC should be purified from the SVF before cell culture. This would provide standardization and help reduce the considerable inconsistency in the current literature regarding reported cell surface markers of ASC, allowing accelerated development of clinical applications.

Differentiation of adipose derived stem cells for clinical applications

The pluripotent ASC can be differentiated into most mesenchymal cell types, including adipocytes,[1,5,6,8–12] osteoblasts,[1,5,6,9–12] chondrocytes,[1,5,6,9,11,12] and myocytes.[1,6,11,12] Because adipose cells are

© 2009 The Authors
Journal compilation © 2009 Royal Australasian College of Surgeons

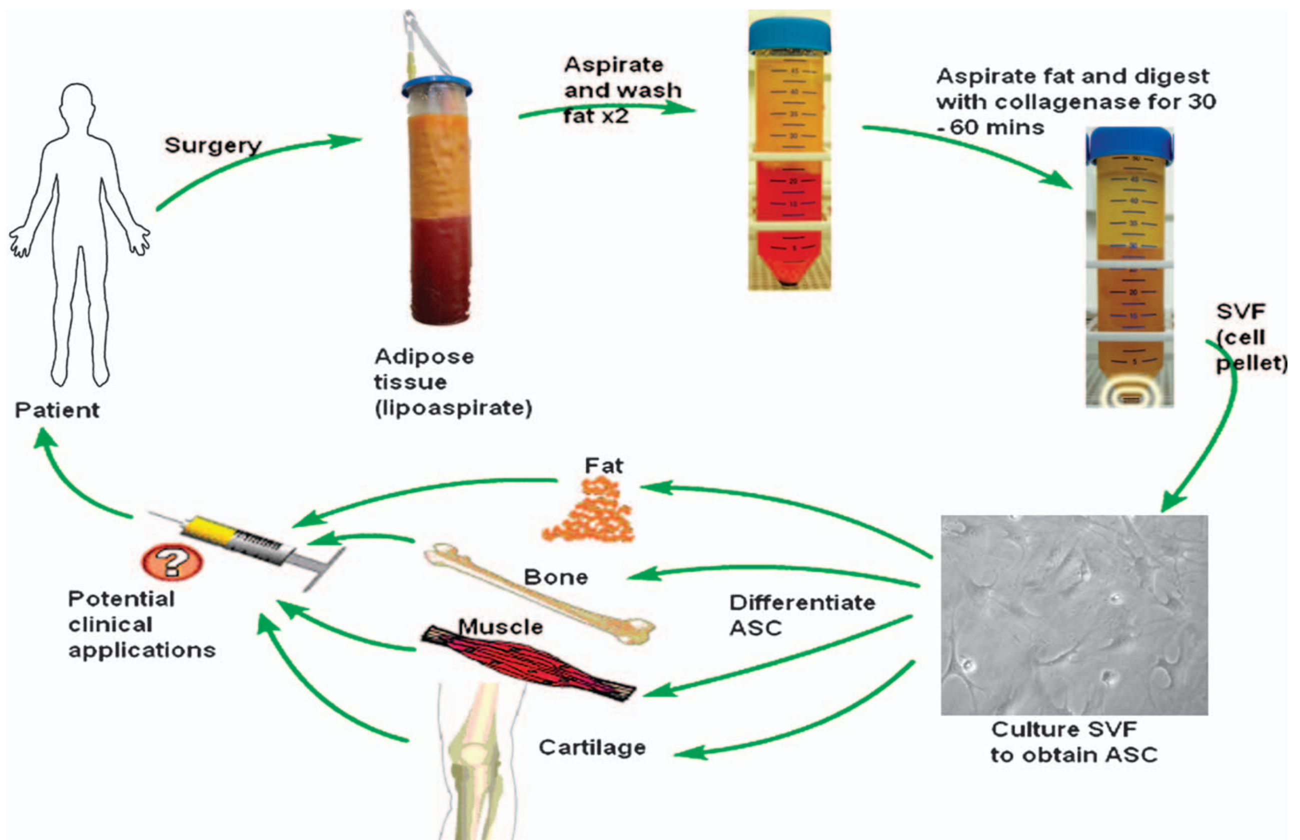


Fig. 1. Summary of cycle of human adipose-derived stem cell (ASC) isolation and differentiation for clinical usage. SVF, stromal vascular cell fraction.

mesodermal in origin, the differentiation of adipose stem cells into neural tissue of ectodermal origin has been thought to be very unlikely.[28] When exposed *in vitro* to antioxidants (in the absence of serum), however, cells take on a bipolar morphology similar to neuronal cells,[6,12,14,30,33] although confirmation of this cell type by any neurophysiological testing has yet to be performed. It also appears that ASC can provide angiogenic[34,35] and possibly haematopoietic support.[11,12] Limited clinical studies have been undertaken with ASC, but current data on these functions are restricted to case reports and early phase 1 trials.

Adipogenesis

Adipogenesis may have many uses in soft-tissue reconstruction in humans. Thousands of patients undergo fat grafting to improve contour defects and wrinkles. In the USA 90 642 fat injections were performed by members of the American Society of Aesthetic Plastic Surgeons in 2005.[20] Fat grafting may rely not only on transplantation of mature adipocytes but also on the ASC fraction, which has the ability to both differentiate into adipocytes and support new blood vessel growth.[28] Purification of ASC and enhanced re-injection of these could improve long-term survival of fat grafts by encouraging adipogenesis and angiogenesis.

Large soft-tissue defects are also common, following trauma and surgical resection (such as mastectomy). In reconstructive plastic surgery, adipose tissue represents a major component of the tissue defect.[36,37] Reconstruction of a large defect, however, involves unique problems to overcome, largely related to angiogenesis and long-term maintenance of volume in engineered grafts.[16,37] It may be possible for tissue engineering-based techniques, such as the use of microchannelled hydrogel cylinders[38] or macroporous hydrogel fragments,[39] to help repair these defects.

A single study looking at lipodystrophy has shown that this condition may be cured in a murine model by adipose transplantation.[40] While lipodystrophy used to be a rare condition, it is now seen more often because it is associated with HIV-positive patients on highly active antiretroviral therapy, due to protease inhibitor interference with lipid metabolism.[41,42] ASC may therefore have a role in the treatment of this difficult condition. It must also be noted that identification of molecules that are expressed solely in differentiating ASC could potentially enable their use as drug targets for the prevention of adipogenesis in obesity and related diseases such as diabetes mellitus or cardiovascular disease.[43,44]

In the laboratory, differentiation into adipocytes takes 14 days, after which time intracellular lipid can be seen after staining with

© 2009 The Authors
Journal compilation © 2009 Royal Australasian College of Surgeons

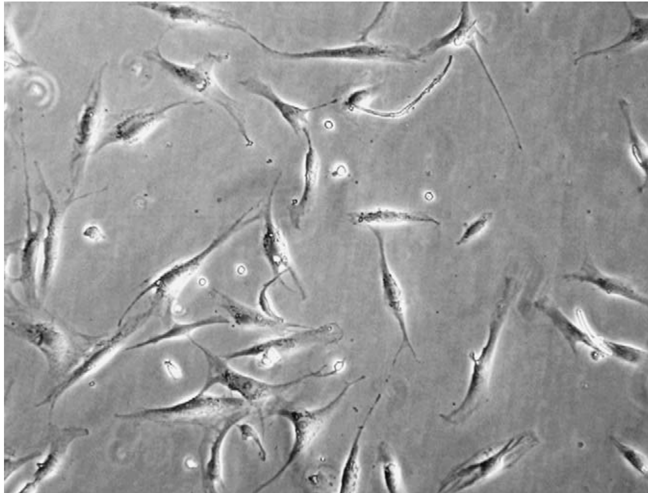


Fig. 2. Culture containing adipose-derived stem cells 2 days after isolation of the stromal vascular fraction (SVF) from human lipoaspirate. At confluence, these cells conformed to the cell surface phenotype shown in Table 1 on flow cytometry (data not shown).

Oil Red O stain (Fig. 3). The gene or factor responsible for signalling the multipotent stem cell to the adipocyte lineage *in vivo* is unknown.[45] The most important stimulants, however, appear to be insulin and glucocorticoids. *In vitro*, the first step of adipogenesis is stimulated by insulin-like growth factor-1 (IGF-1). Growth hormone, glucocorticoids, insulin and fatty acids are stimulatory in both the early and late phases of adipocyte differentiation. Triiodothyronine (T3) hormone stimulates the late phase only.[8,10] Consequently, the induction media for adipogenesis is usually supplemented with a cyclic adenosine monophosphate inducer such as isobutylmethylxanthine, as well as the aforementioned factors to induce differentiation.[1,6,10,14,33,46] Fibroblast-like cells lines, such as the 3T3-L1 embryonic murine line, also respond similarly to the same stimuli. Commercially available media, such as that supplied by Zen-Bio,[47] use very similar preparations for both human and murine adipogenic differentiation.

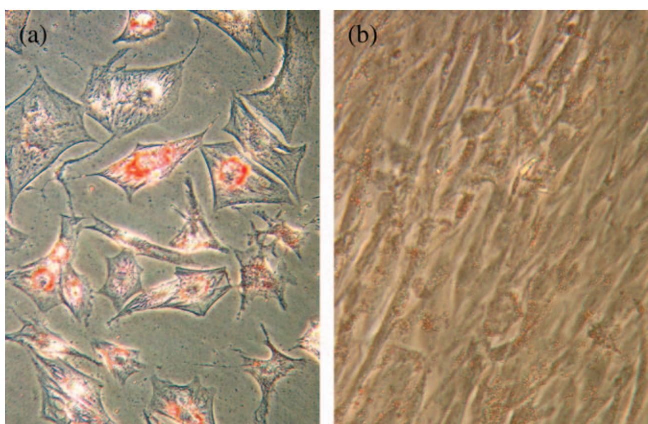


Fig. 3. (a) Differentiation of adipose-derived stem cell (ASC) into adipocytes, as shown by positive Oil Red O staining of intracellular lipid in cells cultured in adipogenic medium for 14 days, not seen in (b) control ASC cultured in complete medium for 14 days.

Retinoids in high concentration, along with cytokines such as interferon, interleukin (IL)-1, IL-2, transforming growth factor-β (TGF-β) and tumour necrosis factor-a are all strongly inhibitory of adipogenesis, so should be avoided in tissue culture protocols designed to induce adipogenesis.[8] It seems that the anti-adipogenic effects of TGF-β are independent of the proliferative effect, and may be mediated in part by inhibition of adipogenic agents such as IGF-1.[8] In contrast, applications of angiogenic growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor-2 and platelet-derived growth factor-BB have been shown in combination to enhance early angiogenesis and increase adipose tissue growth at 6 weeks in a murine model.[48] This could potentially be applied to human ASC to enhance adipogenesis for clinical use.

Osteogenesis

Clinically, differentiation of ASC into osteoblasts could aid repair of fracture non- or mal-unions, and assist bone grafting or joint fusions. The Lee *et al.* study in 2003 provided the first evidence of *in vivo* bone formation following *in vitro* differentiation of ASC down osteoblastic lineages.[10] They isolated ASC from epididymal adipose tissue in Lewis rats, differentiated the cells, reimplanted them in a subcutaneous pocket, and found convincing evidence of bone at 8 weeks.

In 2004 Cowan *et al.* published their study healing critical-sized mouse calvarial defects with ASC seeded onto apatite-coated, polylactic-co-glycolic acid scaffolds.[49] They found 70–90% healing of the defect at 12 weeks, with the results being identical between BMSC and ASC. Chromosomal analysis of the new bone at 2 weeks confirmed that 96–99% of the new bone was from implanted female donor cells, rather than the male recipients. Given that adults, and children over 2 years of age, are unable to reossify large cranial defects, this technology has the potential to heal such wounds. The first such case report in a human was published in 2004.[50] It described a 7-year-old girl with extensive calvarial bone resorption following severe head injury and decompressive craniotomy a year earlier, leaving a cranial defect of approximately 120 cm^2. She was treated with 15 mL cancellous bone graft from the ilium, supplemented with 10 mL ASC that was harvested and isolated during the operative procedure (cell processing took 2 h). Autologous fibrin glue and absorbable Macropore sheets were used for stability. Computed tomography at 3 months showed significant reossification of the defect areas, allowing removal of the protective helmet she had worn for the previous year. This appeared to be safe and efficacious, but obviously further human trials are needed to confirm this result.

© 2009 The Authors
Journal compilation © 2009 Royal Australasian College of Surgeons

Most studies agree that dexamethasone is a requirement for stimulation of osteogenesis *in vitro*,[1,10,33,46] although its exact mechanism of action is yet unknown, and one study replaces this factor with 1,25-dihydroxyvitamin D3.[6] Ascorbic acid functions as a co-factor in the hydroxylation of proline and lysine residues in collagen, and increases the synthesis of non-collagenous bone matrix proteins. Also required is β-glycerophosphate, which is essential for calcification and mineralization of the extracellular matrix.[51] Therefore to stimulate osteogenesis (bone formation), the medium is supplemented with ascorbate derivatives and β-glycerophosphate, along with either dexamethasone or vitamin D.[1,6,10,33,46] One group has studied brief (15-min) incubation of goat ASC with the growth factor bone morphogenetic protein 2 (a member of the TGF-β superfamily of polypeptides), and have shown this to be capable of stimulating osteogenic differentiation in ASC.[52] If applicable to human ASC, such brief pretreatment would be attractive for developing clinical protocols that entailed minimal culture of ASC before reintroduction. *In vitro*, osteogenic differentiation takes at least 28 days, after which time calcified extracellular matrix can be seen after staining with 2% Alizarin red stain (Fig. 4).

Chondrogenesis

If worthwhile chondrogenesis could be achieved, ASC could be used to treat traumatized or arthritic joints, or aid in joint reconstruction. ASC have been used to heal rabbit osteochondral defects.[53] A punch was used to create a 6 × 3 × 0.5-mm defect in the medial femoral condyles. The treatment side received fibrin and ASC (transfected with *lacZ* gene), and these were compared with periosteal-cell derived or native repair mechanisms. Results showed that, at all time periods, repair was better with the ASC cells than on the control side, although no treatment repaired the cartilage back to an undamaged level of function. Given the inherent difficulties in stimulating cartilage regeneration *in vivo*, these early results show promise, even though there are no reports yet of the growth of cartilage to a physiological biomechanical standard.

In the laboratory, chondrogenic differentiation is induced by the addition of insulin, TGF-β1 and ascorbate (with or without dexamethasone) to the medium.[1,6,33,54] Enhanced expression of the extracellular matrix proteins of cartilage occurs when the cells are suspended in a 3-D calcium alginate gel, allowing collagen type II, type IV and aggrecan to develop for several weeks. High-density cell culture ('micromass') techniques also help imitate *in vivo* conditions, and facilitate the phenotypic change of the cells into dense chondrogenic nodules (Fig. 5).[6] When cells are in a monolayer, genetic markers of differentiation are present but in reduced concentration.[54]

Despite this, chondrogenesis does not yet seem to be efficient in cultured ASC. Winter *et al.* performed a comparative study of BMSC and ASC, which showed that the ASC were less sensitive to chondroinductive culture manipulation, and over 2 weeks their differentiation was less complete than BMSC.[54] Gene expression analysis after chondrogenic induction also suggested that there was a qualitative difference between the two, which become apparent in 3-D cell culture, as BMSC responded to 3-D culture techniques with improved chondrogenesis.[54] In that study, differentiation to a molecular phenotype similar to that of cartilage was only achieved in BMSC not ASC.

Myogenesis

Smooth and skeletal muscle has reportedly been generated by differentiation of the ASC in complete media with 5% horse serum, and glucocorticoid such as hydrocortisone and/or dexamethasone.

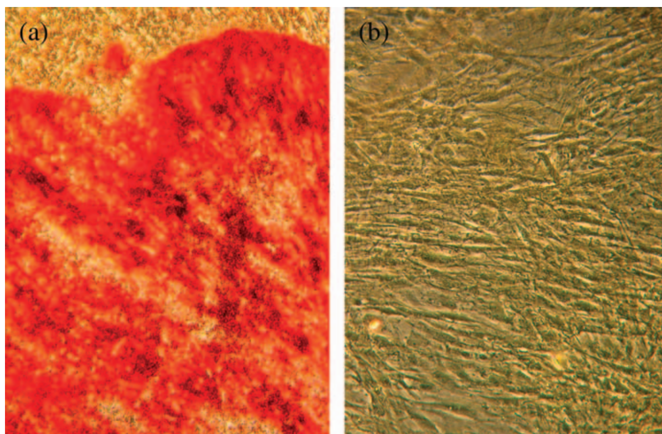


Fig. 4. (a) Differentiation of adipose-derived stem cell (ASC) into osteoblasts, as shown by positive 2% Alizarin red staining of calcified extracellular matrix in cells cultured in osteogenic medium for 38 days, not seen in (b) control ASC cultured in complete medium for 38 days.

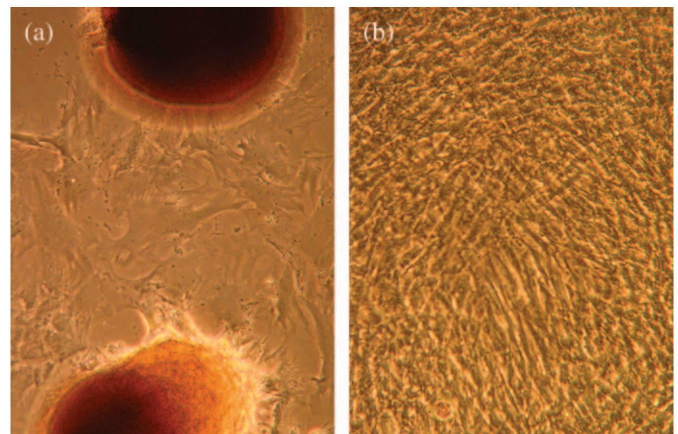


Fig. 5. (a) Differentiation of adipose-derived stem cell (ASC) into dense chondrogenic nodules, as shown in cells plated at high density using a micromass technique and cultured in chondrogenic medium for 14 days, not seen in (b) similarly plated control ASC cultured in complete medium for 14 days.

© 2009 The Authors
Journal compilation © 2009 Royal Australasian College of Surgeons

When cultured under these conditions, cells have been reported to fuse and form multi-nucleated myotubes, yielding protein markers for myocytes.[1,6,33] Myocyte differentiation, however, occurs with the lowest yield and reproducibility of all the differentiated cell types in the literature.[28]

Rodriguez *et al.* transplanted normal human ASC into the tibialis anterior muscle of dystrophin-deficient immunocompetent mice (an animal model of Duchennes muscular dystrophy).[55] The injected ASC survived and integrated into the tibialis muscle. Dystrophin was detected in 50% of the treated myofibers 10 days after injection, and 90% at 6 months. Interestingly, by 50 days, dystrophin-positive myofibers were seen in the adjacent gastrocnemius muscle, indicating cell migration from the injection site to other dystrophic muscles.

Haematopoietic and vascular uses

The literature on the utility of ASC in supporting angiogenesis is often difficult to interpret due to the mixed cell populations present in the cell preparations derived from adipose tissue by different groups. Early work in the 1990s on analysis of lipoaspirate concluded that endothelial cells were the major cell type in the SVF, not stem cells, on the basis of vWF staining,[56] as well as the expression of endothelial cell-specific antigen EN4, which has since been found to react with the CD31 antigen. Porcine properitoneal fat containing such cells was cultured and used to seed polytetrafluoroethylene prosthetic vascular grafts.[4] That study showed that seeding of endothelial cells onto the graft did occur, but because those studies used the entire SVF in their experiments, the origin of the endothelial cells may have been endothelial cells or precursors in the SVF rather than from ASC. Future work is needed to determine whether ASC can be used to improve patency and the long-term results of vascular grafts.[12]

More recently, studies have looked at cell surface markers, usually on flow cytometry, to define the presence and contribution of different cell lineages to the angiogenic properties of the SVF. ASC are not considered to express differentiated haematopoietic cell markers, such as CD14, CD16, CD56, human leukocyte antigen-DR/major histocompatibility complex (HLA-DR/MHC) class II and CD45, nor markers shared by leucocytes and endothelial cells such as CD31.[6,9,14,57,58] In contrast, studies looking at embryonic stem cells support the theory that endothelial precursor cells are $CD31^+$.[59,60] Therefore the $CD31^+$ cells commonly seen in SVF[31] and early passage cultures may be endothelial precursors that account for some of the endothelial growth reported in earlier studies.[4,56]

Expression of CD34, which is known to be expressed by haematopoietic stem cells, has been controversial. Although CD34 was not initially considered a marker of ASC, well-established ASC research groups have subsequently shown its presence to varying degrees.[2,5] It now seems likely that ASC are $CD34^+$ in SVF preparations, but that this marker is subsequently lost during *in vitro* culture.[31]

ASC express and secrete many haematopoietic cell cytokines, including macrophage colony-stimulating factor and granulocyte–macrophage colony-stimulating factor.[12] There is also good evidence that they secrete both anti-apoptotic and angiogenic factors, including VEGF, hepatocyte growth factor and TGF-β.[35,61] Under hypoxic conditions, there is a significant increase in the secretion of VEGF, which stimulates increased endothelial cell numbers with lower rates of apoptosis.[35] Using an *in vivo* model of hind limb ischaemia in nude mice, injection of ASC has been shown to markedly improve limb perfusion, associated with increased blood flow and capillary density in the limb.[34,35] While incorporation of ASC into the neovasculature has been suggested to account for some of these changes, we believe that the weight of evidence supports the conclusion that the role of ASC in angiogenesis relates to the enhancement of angiogenesis through secretion of factors such as VEGF, rather than true differentiation by ASC into endothelial cells.

Given that chronic tissue ischaemia is the presumed pathogenesis of late complications of radiotherapy, a recently published human study treated 20 consecutive patients with radiotherapy-related complications such as fibrosis and osteoradionecrosis of the chest wall with traditional fat graft injections of autologous lipoaspirates.[58] All patients in the study had progressive tissue regeneration, including neovessel formation and improved hydration. While impressive clinical results were achieved, the use of standard fat grafting techniques again means that the role of ASC remains uncertain, because the lipoinjections would have included many other cells types. Nevertheless, this form of therapy potentially represents a practical, minimally invasive treatment for microangiopathies.

To assess the haematopoietic function of ASC, a small animal study published in 2003 attempted to reconstitute lethally irradiated mice using ASC rather than BMSC.[57] A total of 40% of lethally irradiated mice could be salvaged with same-day intraperitoneal (i.p.) injection of ASC. This compared favourably with both i.p. injection of BMSC (which also had a 40% salvage rate). Reconstitution was slower with ASC than BMSC, however, and although intravenous (i.v.) injection was the most effective method of BMSC transplantation, i.v. injection of ASC was fatal. Further analysis suggested that the effect of the ASC was to promote the proliferation of pluripotent haematopoietic stem cells, thus supporting reconstitution of endogenous haematopoiesis.[57]

Other applications

Given all the possibilities for use of ASC, the first phase I clinical trial of ASC therapy was perhaps in a surprising context: the healing of chronic fistulae in Crohn's disease.[62] Following their success in healing a Crohn's rectovaginal fistula with autologous ASC in 2003,[63] a phase I clinical trial was undertaken by a group at the La Paz University Hospital in Madrid.[62] Nine fistulae in four patients were inoculated with ASC, which had been cultured for between 6 and 31 days. Of the eight fistulae followed up weekly, six (75%) showed complete healing within 8 weeks, despite failing medical and classic surgical treatment at least twice preoperatively. No adverse effects were identified, and the authors conclude that the practice is safe and potentially effective, justifying progression to phase II clinical trials.

© 2009 The Authors
Journal compilation © 2009 Royal Australasian College of Surgeons

Human ASC injected into immunosuppressed mice engraft into many organs, including bone marrow, brain, thymus, heart, liver, and lung,[46,64] suggesting that they may have potential to aid repair of many organs. They exhibit phagocytosis in culture medium,[65] which may be a useful trait in tissue repair. Potential applications outside the normal roles expected for mesenchymal cells have also been reported: one group has reported differentiation of ASC into cells with a pancreatic endocrine phenotype, with potential for treating diabetes.[66] ASC might also be a useful target for therapeutic gene transfer. Recent experiments have shown that ASC are able to be transduced with both lentivirus and replication-defective Herpes simplex virus (HSV).[55,67] Both herpesvirus entry mediated A (HveA) and HveC HSV receptors have been found on human ASC and mature adipocytes, making them excellent targets for HSV-based vectors.

Barriers to further clinical applications

One of the biggest issues concerning ASC literature at present is the lack of researcher standardization, with most groups using bulk culture of adipose-derived SVF cells to initiate ASC culture. As noted previously, although SVF is a heterogeneous mixture of cells, only a small proportion of these cells subsequently adhere to plastic tissue cultureware and proliferate. Even so, in the absence of definitive markers of ASC, it remains possible that many experiments reported in the literature used mixed populations of cells that included, but did not exclusively consist of, ASC.

Definitive cell surface markers of ASC would help not only distinguish them from other cell populations in cell culture, but would also enable purification of ASC from SVF. Several markers for BMSC[68] have been examined for their suitability as ASC markers also, such as STRO1 and CD73,[1,6,69] but as yet there is no consensus on their relationship to ASC and neither appear to be a unique identifier. Consequently, ASC can be unequivocally identified only by functional assays[70] or in retrospect, once they undergo differentiation.

Over the past 6 years many papers have reported characterization of cell surface markers at different stages of SVF or ASC culture. Some proteins are consistently identified on the ASC surface by different groups,[12,31,33] and it appears that ASC and BMSC have similar, although not identical, surface protein profiles.[2,5,12,69,71] There is considerable heterogeneity, however, in the full range of ASC cell surface markers reported. Some of this heterogeneity is found in the cell populations within a single culture, indicating either the presence of mixed populations of cells, or the modulation of cell surface proteins during cell culture. In this respect it is important to note that several of the cell surface markers commonly analysed are highly susceptible to modulation by cell culture in plastic tissue cultureware, for example, adhesion molecules. Heterogeneity is also apparent when comparing the reports from different groups, suggesting that subtle differences in the cell purification or culture procedures, or in the timing of the analyses relative to manipulation *in vitro*, are influencing the reported cell phenotype. Different groups also summarize their results in variable ways or use different thresholds to reporting positive staining, complicating comparisons between studies.[5,6,9] Taking the literature as a whole, we have summarized the cell surface proteins expressed by ASC according to the consistency of the evidence, as shown in Table 1.

Relatively few papers report sorting of different populations from SVF and subsequent determination of whether these populations contained ASC. We feel that this approach would provide the best

Table 1 Characterization of surface markers of ASC according to the current literature

Consistently expressed	References	Consistently not expressed	References	Variably reported	References
CD9	5	CD11a	5,72	CD34†	2,5,6,17,31,69,71,73
CD10	5,14	CD11b	5,14,72	CD49b‡	31,72
CD13‡	2,5,6,14,17,31,69,73	CD11c	5,69,72	CD49d‡	6,31,69,72
CD29‡	5,6,14,17,31,69,72	CD14	5,6,69,73	CD54	5,69
CD44‡	2,5,6,14,17,31,69	CD16	6,69	CD61	6,72
CD49e	5,14,31,72	CD18	5,72	CD62e	6
CD51	31,72	CD31†	6,31,69	CD63‡	17,31
CD55	5,73	CD45	2,6,14,31,69	CD71	6
CD59	5,14,73	CD50	5	CD73‡	2,6,17,69
CD90‡	2,14,17,31,69,72	CD56	5,6,69	CD105‡	2,5,6,17,31,69
CD166‡	5,14,69	CD104	6,31,69	CD106	6,69,72
		HLA-DR	2,5,14,31,72	CD140a	72
				CD146	17,31

†Despite the variable reports in the literature it now seems likely that the ASC reside in the $CD34^+$ $CD31^-$ fraction of the SVF, but that CD43 is lost with subsequent culture.[31,74]
‡Marker consistently upregulated with culture.[17,31] ASC, adipose-derived stem cell; SVF, stromal vascular fraction.

© 2009 The Authors
Journal compilation © 2009 Royal Australasian College of Surgeons

evidence of the cell surface phenotype of ASC. More work is needed on the molecular characteristics of ASC to allow their earlier identification. Cell-sorting experiments carried out immediately after isolation of the SVF from adipose tissue would help discriminate between markers that identify ASC *in vivo*, and those that are expressed only after *in vitro* manipulation. There is early evidence that the cells in the SVF that are $CD34^+$ and $CD31^-$ are the population with the most characteristics of ASC.[31,74] Standardization of techniques among research groups would reduce the disparity in current reports, which would improve the clinical usefulness of ASC, as well as help avoid the potential laboratory artefacts inherent in using impure cell populations in research experimentation.

Future directions

Clearly, a number of challenges remain before ASC can be used in everyday clinical practice. Discovery of specific markers for ASC will ultimately accelerate research, and potentially allow rapid purification of ASC, perhaps enabling immediate use in some circumstances without subsequent cell culture. Reliable, fast, and efficient protocols for differentiation of ASC are also needed. At present adipogenesis is relatively fast, occurring in approximately 2 weeks, but chondrogenesis and osteogenesis take significantly longer and cell yields are still low, despite these differentiation protocols including several factors similar to the adipogenesis protocols, such as steroids. Any improvement in these differentiation timeframes will make ASC more attractive for tissue engineering purposes. This may be achievable by the pretreatment of cells with various cytokines or growth factors. Identification of useful, reliable matrices for 3-D culture of the aforementioned cells lines would be clinically useful. Knowledge of surface receptors, and therefore likely responses to soluble factors and extracellular matrix components, may be used to expedite *in vitro* culture. Because ASC are currently cultured in media containing bovine serum, there is a theoretical risk of transmission of infection to human ASC from the culture media with commonly used cell culture protocols. The ability to differentiate the cell lines in serum free media, currently being explored in at least one laboratory in the USA,[16] would also be desirable to reduce the risk of contamination of cells prior to clinical use. Consistent and reliable *in vivo* results in animal models must also be confirmed, to ensure safety and reliability of ASC implantation.

The ability to reliably cryopreserve and recover ASC over long time periods would also be useful. One study has shown that the best post-thaw membrane integrity rates are achieved with slow cooling rates and higher end temperatures,[75] but because the hold time (time spent at the end temperature) was only 15 min in that study, clearly more research needs to be done with time frames closer to clinical usage (days–months).

Despite the occasional case report supporting safety of ASC in humans,[50,63] large-scale human trials will be needed to confirm safety as well as efficacy. Some recent studies have found immunosuppressive properties in implanted ASC,[2,55] as for BMSC.[68] Although local immunosuppression may be a useful property in some settings, it clearly has potential risks, such as allowing occult cancers to evade immune surveillance.[28] Only large-scale trials will clarify the true risk/benefit ratio of ASC therapy.

Despite these comments, many of these issues are currently being addressed, and rapid progress is expected. Given the ease of harvest, isolation and culture of ASC, as well as their relative abundance, it is likely that ASC will find widespread application in clinical practice in the future.

Acknowledgements

The principal author gratefully acknowledges support from the Royal Australasian College of Surgeons, for the granting of the Foundation Research Award, the New Zealand Research Fellowship and the Plastic and Reconstructive Surgery Research Award. Dr Michelle Locke is the recipient of research grants the Royal Australasian College of Surgeons, namely the Foundation Research Award 2007, the New Zealand Research Fellowship 2008 and the Plastic and Reconstructive Surgery Research Award. The authors also gratefully acknowledge the Stevenson Charitable Trust for support of research costs in the production of this work.

References

1. Zuk PA, Zhu M, Mizuno H *et al*. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng*. 2001; **7**: 211–28.
2. Puissant B, Barreau C, Bourin P *et al*. Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. *Br. J. Haematol*. 2005; **129**: 118–29.
3. Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* 2006; **24**: 1294–301.
4. Young C, Jarrell BE, Hoying JB, Williams SK. A porcine model for adipose tissue-derived endothelial cell transplantation. *Cell Transplant*. 1992; **1**: 293–8.
5. Gronthos S, Franklin DM, Leddy HA, Robey PG, Storms RW, Gimble JM. Surface protein characterization of human adipose tissue-derived stromal cells. *J. Cell Physiol*. 2001; **189**: 54–63.
6. Zuk PA, Zhu M, Ashjian P *et al*. Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell*. 2002; **13**: 4279–95.
7. Short B, Brouard N, Occhiodoro-Scott T, Ramakrishnan A, Simmons PJ. Mesenchymal stem cells. *Arch. Med. Res*. 2003; **34**: 565–71.
8. Boone C, Mourot J, Gregoire F, Remacle C. The adipose conversion process: regulation by extracellular and intracellular factors. *Reprod. Nutr. Dev*. 2000; **40**: 325–58.
9. Wickham MQ, Erickson GR, Gimble JM, Vail TP, Guilak F. Multipotent stromal cells derived from the infrapatellar fat pad of the knee. *Clin. Orthop. Relat. Res*. 2003; (**412**): 196–212.
10. Lee JA, Parrett BM, Conejero JA *et al*. Biological alchemy: engineering bone and fat from fat-derived stem cells. *Ann. Plast. Surg*. 2003; **50**: 610–17.
11. Gimble J, Guilak F. Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. *Cytotherapy* 2003; **5**: 362–9.

© 2009 The Authors
Journal compilation © 2009 Royal Australasian College of Surgeons

12. Gimble JM, Guilak F. Differentiation potential of adipose derived adult stem (ADAS) cells. *Curr. Top. Dev. Biol*. 2003; **58**: 137–60.
13. Zuk PA. Consensus statement. In: *International Fat Applied Technology Society 2nd International Meeting*. Pittsburg, PA, 2004.
14. Aust L, Devlin B, Foster SJ *et al*. Yield of human adipose-derived adult stem cells from liposuction aspirates. *Cytotherapy* 2004; **6**: 7–14.
15. Boquest AC, Shahdadfar A, Brinchmann JE, Collas P. Isolation of stromal stem cells from human adipose tissue. *Methods Mol. Biol*. 2006; **325**: 35–46.
16. Parker AM, Katz AJ. Adipose-derived stem cells for the regeneration of damaged tissues. *Expert Opin. Biol. Ther.* 2006; **6**: 567–78.
17. Mitchell JB, McIntosh K, Zvonic S *et al*. Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. *Stem Cells* 2006; **24**: 376–85.
18. Sekiya I, Larson BL, Smith JR, Pochampally R, Cui J-G, Prockop DJ. Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. *Stem Cells* 2002; **20**: 530–41.
19. Sommer B, Sattler G. Current concepts of fat graft survival: histology of aspirated adipose tissue and review of the literature. *Dermatol. Surg*. 2000; **26**: 1159–66.
20. American Society for Aesthetic Plastic Surgery (ASAPS). *Cosmetic Surgery Quick Facts: 2005 ASAPS Statistics*. New York, NY: ASAPS, 2006. [Cited 12 January 2007.] Available from URL: http://surgery.org/press/procedurefacts-asqf.php.
21. Hanke CW, Coleman WP III. Morbidity and mortality related to liposuction. Questions and answers. *Dermatol. Clin*. 1999; **17**: 899–902.
22. Housman TS, Lawrence N, Mellen BG *et al*. The safety of liposuction: results of a national survey. *Dermatol. Surg*. 2002; **28**: 971–8.
23. Yoho RA, Romaine JJ, O'Neil D. Review of the liposuction, abdominoplasty, and face-lift mortality and morbidity risk literature. [Erratum appears in *Dermatol. Surg*. 2005; **31** (9 Pt 1): 1158]. *Dermatol. Surg*. 2005; **31**: 733–43; discussion 43.
24. Rohrich RJ, Beran SJ. Is liposuction safe? *Plast. Reconstr. Surg*. 1999; **104**: 819–22.
25. Shiffman MA, Mirrafati S. Fat transfer techniques: the effect of harvest and transfer methods on adipocyte viability and review of the literature. *Dermatol. Surg*. 2001; **27**: 819–26.
26. Rodbell M. Metabolism of isolated fat cells. Effects of hormone on fat metabolism and lipolysis. *J. Biol. Chem*. 1964; **223**: 375–80.
27. Deslex S, Negrel R, Vannier C, Etienne J, Ailhaud G. Differentiation of human adipocyte precursors in a chemically defined serum-free medium. *Int. J. Obes*. 1987; **11**: 19–27.
28. Tholpady SS, Llull R, Ogle RC, Rubin JP, Futrell JW, Katz AJ. Adipose tissue: stem cells and beyond. *Clin. Plast. Surg*. 2006; **33**: 55–62.
29. Ogawa R. The importance of adipose-derived stem cells and vascularized tissue regeneration in the field of tissue transplantation. *Curr. Stem Cell Res. Ther.* 2006; **1**: 13–20.
30. Safford KM, Rice HE. Stem cell therapy for neurologic disorders: therapeutic potential of adipose-derived stem cells. *Curr. Drug. Targets* 2005; **6**: 57–62.
31. Boquest AC, Shahdadfar A, Fronsdal K *et al*. Isolation and transcription profiling of purified uncultured human stromal stem cells: alteration of gene expression after in vitro cell culture. *Mol. Biol. Cell*. 2005; **16**: 1131–41.
32. Wosnitza M, Hemmrich K, Groger A, Graber S, Pallua N. Plasticity of human adipose stem cells to perform adipogenic and endothelial differentiation. *Differentiation* 2007; **75**: 12–23.
33. Gimble JM. Adipose tissue-derived therapeutics. *Expert. Opin. Biol. Ther.* 2003; **3**: 705–13.
34. Miranville A, Heeschen C, Sengenes C, Curat CA, Busse R, Bouloumie A. Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. *Circulation* 2004; **110**: 349–55.
35. Rehman J, Traktuev D, Li J *et al*. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* 2004; **109**: 1292–8.
36. Brey EM, Patrick CW Jr. Tissue engineering applied to reconstructive surgery. *IEEE Eng. Med. Biol. Mag*. 2000; **19**: 122–5.
37. Stosich MS, Mao JJ. Adipose tissue engineering from human adult stem cells: clinical implications in plastic and reconstructive surgery. *Plast. Reconstr. Surg*. 2007; **119**: 71–83; discussion 4–5.
38. Stosich MS, Bastian B, Marion NW, Clark PA, Reilly G, Mao JJ. Vascularized adipose tissue grafts from human mesenchymal stem cells with bioactive cues and microchannel conduits. *Tissue Eng*. 2007; **13**: 2881–90.
39. Halberstadt C, Austin C, Rowley J *et al*. A hydrogel material for plastic and reconstructive applications injected into the subcutaneous space of a sheep. *Tissue Eng*. 2002; **8**: 309–19.
40. Gavrilova O, Marcus-Samuels B, Graham D *et al*. Surgical implantation of adipose tissue reverses diabetes in lipoatrophic mice. *J. Clin. Invest*. 2000; **105**: 271–8.
41. Carr A, Samaras K, Burton S *et al*. A syndrome of peripheral lipodystrophy, hyperlipidaemia and insulin resistance in patients receiving HIV protease inhibitors. *AIDS* 1998; **12**: F51–8.
42. Davison SP, Timpone J Jr, Hannan CM. Surgical algorithm for management of HIV lipodystrophy. *Plast. Reconstr. Surg*. 2007; **120**: 1843–58.
43. Rodriguez A-M, Elabd C, Delteil F *et al*. Adipocyte differentiation of multipotent cells established from human adipose tissue. *Biochem. Biophys. Res. Commun*. 2004; **315**: 255–63.
44. Lee H-K, Lee B-H, Park S-A, Kim C-W. The proteomic analysis of an adipocyte differentiated from human mesenchymal stem cells using two-dimensional gel electrophoresis. *Proteomics* 2006; **6**: 1223–9.
45. Otto TC, Lane MD. Adipose development: from stem cell to adipocyte. *Crit. Rev. Biochem. Mol. Biol*. 2005; **40**: 229–42.
46. Kim DH, Je CM, Sin JY, Jung JS. Effect of partial hepatectomy on in vivo engraftment after intravenous administration of human adipose tissue stromal cells in mouse. *Microsurgery* 2003; **23**: 424–31.
47. Zen-Bio. *Zen-Bio Product Manuals Human Cell Systems and Assay Kits*. Durham, NC: Zen-Bio Inc., 2007. [Cited 27 July 2007.] Available from URL: http://www.zen-bio.com/download.html
48. Rophael JA, Craft RO, Palmer JA *et al*. Angiogenic growth factor synergism in a murine tissue engineering model of angiogenesis and adipogenesis. *Am. J. Pathol*. 2007; **171**: 2048–57.
49. Cowan CM, Shi Y-Y, Aalami OO *et al*. Adipose-derived adult stromal cells heal critical-size mouse calvarial defects. *Nat. Biotechnol*. 2004; **22**: 560–7.
50. Lendeckel S, Jodicke A, Christophis P *et al*. Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: Case report. *J. Craniomaxillofac. Surg*. 2004; **32**: 370–3.
51. Duplomb L, Dagouassat M, Jourdon P, Heymann D. Differentiation of osteoblasts from mouse embryonic stem cells without generation of embryoid body. *In Vitro Cell. Dev. Biol. Anim*. 2007; **43**: 21–4.
52. Knippenberg M, Helder MN, Zandieh Doulabi B, Wuisman PIJM, Klein-Nulend J. Osteogenesis versus chondrogenesis by BMP-2 and BMP-7 in adipose stem cells. *Biochem. Biophys. Res. Commun*. 2006; **342**: 902–8.

© 2009 The Authors
Journal compilation © 2009 Royal Australasian College of Surgeons

53. Nathan S, Das De S, Thambyah A, Fen C, Goh J, Lee EH. Cell-based therapy in the repair of osteochondral defects: a novel use for adipose tissue. *Tissue Eng*. 2003; **9**: 733–44.
54. Winter A, Breit S, Parsch D *et al*. Cartilage-like gene expression in differentiated human stem cell spheroids: a comparison of bone marrow-derived and adipose tissue-derived stromal cells. *Arthritis Rheum*. 2003; **48**: 418–29.
55. Rodriguez A-M, Pisani D, Dechesne CA *et al*. Transplantation of a multipotent cell population from human adipose tissue induces dystrophin expression in the immunocompetent mdx mouse. *J. Exp. Med*. 2005; **201**: 1397–405.
56. Williams SK, Wang TF, Castrillo R, Jarrell BE. Liposuction-derived human fat used for vascular graft sodding contains endothelial cells and not mesothelial cells as the major cell type. *J. Vasc. Surg*. 1994; **19**: 916–23.
57. Cousin B, Andre M, Arnaud E, Penicaud L, Casteilla L. Reconstitution of lethally irradiated mice by cells isolated from adipose tissue. *Biochem. Biophys. Res. Commun*. 2003; **301**: 1016–22.
58. Rigotti GMD, Marchi AMD, Galie MPD *et al*. Clinical treatment of radiotherapy tissue damage by lipoaspirate transplant: a healing process mediated by adipose-derived adult stem cells. *Plast. Reconstr. Surg*. 2007; **119**: 1409–22.
59. Redick SD, Bautch VL. Developmental platelet endothelial cell adhesion molecule expression suggests multiple roles for a vascular adhesion molecule. *Am. J. Pathol*. 1999; **154**: 1137–47.
60. Li ZJ, Wang ZZ, Zheng YZ *et al*. Kinetic expression of platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) during embryonic stem cell differentiation. *J. Cell. Biochem*. 2005; **95**: 559–70.
61. Cao Y, Sun Z, Liao L, Meng Y, Han Q, Zhao RC. Human adipose tissue-derived stem cells differentiate into endothelial cells in vitro and improve postnatal neovascularization in vivo. *Biochem. Biophys. Res. Commun*. 2005; **332**: 370–9.
62. Garcia-Olmo D, Garcia-Arranz M, Herreros D, Pascual I, Peiro C, Rodriguez-Montes JA. A phase I clinical trial of the treatment of Crohn's fistula by adipose mesenchymal stem cell transplantation. *Dis. Colon Rectum* 2005; **48**: 1416–23.
63. Garcia-Olmo D, Garcia-Arranz M, Garcia LG *et al*. Autologous stem cell transplantation for treatment of rectovaginal fistula in perianal Crohn's disease: a new cell-based therapy. *Int. J. Colorectal Dis*. 2003; **18**: 451–4.
64. Kang SK, Lee DH, Bae YC, Kim HK, Baik SY, Jung JS. Improvement of neurological deficits by intracerebral transplantation of human adipose tissue-derived stromal cells after cerebral ischemia in rats. *Exp. Neurol*. 2003; **183**: 355–66.
65. Cousin B, Munoz O, Andre M *et al*. A role for preadipocytes as macrophage-like cells. *FASEB J*. 1999; **13**: 305–12.
66. Timper K, Seboek D, Eberhardt M *et al*. Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin, and glucagon expressing cells. *Biochem. Biophys. Res. Commun*. 2006; **341**: 1135–40.
67. Fradette J, Wolfe D, Goins WF, Huang S, Flanigan RM, Glorioso JC. HSV vector-mediated transduction and GDNF secretion from adipose cells. *Gene. Ther*. 2005; **12**: 48–58.
68. Le Blanc K, Tammik C, Rosendahl K, Zetterberg E, Ringden O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp. Hematol*. 2003; **31**: 890–6.
69. De Ugarte DA, Alfonso Z, Zuk PA *et al*. Differential expression of stem cell mobilization-associated molecules on multi-lineage cells from adipose tissue and bone marrow. *Immunol. Lett*. 2003; **89**: 267–70.
70. Rodriguez AM, Elabd C, Amri E-Z, Ailhaud G, Dani C. The human adipose tissue is a source of multipotent stem cells. *Biochimie* 2005; **87**: 125–8.
71. Fraser JK, Wulur I, Alfonso Z, Hedrick MH. Fat tissue: an underappreciated source of stem cells for biotechnology. *Trends Biotechnol*. 2006; **24**: 150–4.
72. Katz AJ, Tholpady A, Tholpady SS, Shang H, Ogle RC. Cell surface and transcriptional characterization of human adipose-derived adherent stromal (hADAS) cells. *Stem Cells* 2005; **23**: 412–23.
73. Festy F, Hoareau L, Bes-Houtmann S *et al*. Surface protein expression between human adipose tissue-derived stromal cells and mature adipocytes. *Histochem. Cell Biol*. 2005; **124**: 113–21.
74. Varma MJO, Breuls RGM, Schouten TE *et al*. Phenotypical and functional characterization of freshly isolated adipose tissue-derived stem cells. *Stem Cells Dev*. 2007; **16**: 91–104.
75. Thirumala S, Zvonic S, Floyd E, Gimble JM, Devireddy RV. Effect of various freezing parameters on the immediate post-thaw membrane integrity of adipose tissue derived adult stem cells. *Biotechnol. Prog*. 2005; **21**: 1511–24.

© 2009 The Authors
Journal compilation © 2009 Royal Australasian College of Surgeons