Adipose-derived stem cells for clinical applications: a review

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Abstract

The use of stem cells derived from adipose tissue as an autologous and self-replenishing source for a variety of differentiated cell phenotypes, provides a great deal of promise for reconstructive surgery. In this article, we review available literature encompassing methods of extraction of pluripotent adipose stem cells (ASCs) from lipoaspirate locations, their storage, options for culture, growth and differentiation, cryopreservation and its effect on stem cell survival and proliferation, and new technologies involving biomaterials and scaffolds. We will conclude by assessing potential avenues for developing this incredibly promising field.

Introduction

The objective of the plastic surgeon in procedures to improve function and cosmesis is always to replace 'like with like'. With the use of synthetic implants in plastic and reconstructive surgery now commonplace, the focus has turned to ameliorating our understanding of and honing techniques for free fat transfer. This is a rapidly expanding and changing field as we seek to eliminate the hurdles of synthetic fillers, such as foreign body reactions, comparatively high infection rates, need for re-operation, and of course, cost.

Free fat transfer has been documented in the literature since as early as 1893, when Franz Neuber used a strip of fat from a patient's upper arm to reconstruct a facial defect caused by bony tuberculosis. He continued this practice, filling scarred defects, observing that graft size appeared to be directly related to longevity and thus, results (1). While Neuber paved the way for autologous fillers, results

were persistently unpredictable. Optimism was further curtailed by Peer in the 1950s, when published data revealed take rates of no more than 50% (2). Since this landmark, a combination of studies has revealed resorption rates of between 20% to 90%. Thus, free fat transfer lost popularity, further eclipsed by increasing use of synthetic and biological fillers.

The arrival of liposuction as a technique brought back favour for free fat transfer, however, this was hampered by a 1987 review by the American Society of Plastic and Reconstructive Surgeons Ad-Hoc Committee on New Procedures. Concerns regarding potential post-operative interference with breast cancer screening and detection were responsible for the opinion being 'unanimous in deploring the use of fat injection in breast augmentation' (22). It would appear, however, that other applications of free fat transfer continued to blossom, particularly in the cosmetics industry.

In plastic surgery, the potential for using adipose stem cells (ASCs) is endless, with a spectrum of treatment areas and types of reconstruction needed. From oncological reconstruction to aesthetic uses, each application can be individually tailored to the patient.

The aim of this paper is to review the existing literature on applications of harvest, purification, characterization and cryopreservation of adipose-derived stem cells (ASCs).

Methods

Multiple PubMed and Medline searches were performed using the key words 'free fat transfer', 'liposculpture', 'Coleman fat transfer', 'fat injection', 'adipose tissue extraction', 'adipose derived stem cells', 'adipose derived stromal cells', 'adipose stem cells', 'pluripotent adipose cells', 'ADSC', 'ASC', 'harvest of adipose stem cells' and purification of adipose stem cells'. These were reviewed for relevance and those articles dated after the year 2000 were used preferentially unless older articles were required to illustrate a specific issue. Institute PhD

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theses and personal communication were also used as sources.

Adipose tissue and ASCs

Fat first develops in utero in the fourth gestational month in humans and is composed of adipocytes in a connective tissue matrix, which is made up of collagen and elastin, stromal cells and neurovascular structures. The most significant obstacle in propulsion of free fat transfer as a reconstructive modality has been persistently unpredictable and often disappointing rates of graft resorption. The theory is that fat lobules are supplied individually via tiny pedicles. Thus, live fat tissue is revascularized at a transplantation site within 48 h, in the meantime being fed by diffused materials from surrounding free plasma. Non-viable tissue at the site is scavenged by macrophages and replaced with fibrotic tissue and cystic changes.

This accounts for Peer's rationalisation in proposing the cell survival theory, which he used to explain free fat graft survival rates being dependent on size of transplanted fat particles and surface area from which these cells could re-establish their blood supply (2,24). This has led more recently to the practice of multiple successive, smaller fat transfer procedures, to maximize results. Build-up of these means that larger volumes can be replenished, and limited invasiveness of the procedure means that while complications can occur, they are relatively rare. However, even these ameliorated techniques still leave a long way to go for generating reliable and reproducible results. Drawbacks include need for multiple operations, usually under general anaesthesia, and uncertainty relating to true final volume of the implant after resorption has taken place.

Cell therapies are being increasingly studied as a way of replacing faulty or depleted cells in tissues of adult human beings in a range of pathological conditions from heart muscle post-myocardial infarction to non-union of complex bone fractures. Recent advances in stem cell research, particularly focusing on adult bone marrow and embryonic stem cells, hold much promise in the field of regeneration and repair. While the former poses limitations in the quantity of available cells, and the latter is plagued with ethical dilemmas, possibility of extracting pluripotent stem cells from fatty tissue has arisen. Aspirated fat is in plentiful supply in many plastic surgery procedures, such as liposuction and liposculpture, and precursor cells can be purified by a variety of processes and enzymatic techniques to obtain the ASC-rich stromal vascular fraction (SVF). This is now studied as a supplement to free fat transfer in order to increase yield (25,26).

Autologous free fat transfer has found many applications in plastic surgery including: reconstruction and

contouring of the breast and nipple, whether post-oncological resection or *de novo*, facial and hand rejuvenation, facial contouring in HIV lipodystrophy; as well as less common uses such as in improvement of function and appearance of irradiated tissues, and correction of asymmetry in Poland's syndrome and Parry-Romberg syndrome; perhaps less crucially, augmentation of calves and buttocks, and even correction of brassiere strap defects (3–21). More recent applications include top-ups after a variety of operations such as mastectomy, whether alone or accompanied by free or pedicled flap reconstruction. Combination of such a wide range of choices renders free fat transfer increasingly applicable to a variety of patients, and has resulted in breast reconstruction surgery becoming the gold standard after post-oncological diagnoses.

ASCs are also currently being applied therapeutically in a variety of disciplines such as orthopaedic surgery, otolaryngology (in particular, vocal fold grafting) (11), neurosurgery, and general and vascular surgeries, to fill defects and enhance healing, as these ASCs have already been demonstrated as multipotent and capable of differentiating into bone, cartilage, fat, skeletal, cardiac myocyte, endothelial and neuronal lineages. They have also shown pertinence in generation of novel, improved-patency vascular grafts and valves (16,17).

Methods of fat cell harvest and refinement have been under intense scrutiny for the past 30 years, and have been surrounded by controversy. Viability of cells obtained after various harvesting and processing techniques has been assessed by several methods. Suga *et al.* (27) have described the three staple tests of fat cell viability: cell staining, XTT (colorimetric) assay and G3PD assay. Their results approximated those of previous studies (28), which revealed 1.5 million adipocytes per gram of adipose tissue. These assays have since been used to investigate effects of processing variables on content of viable adipose stem cells in a given sample.

ASCs are also capable of expressing multiple growth factors including vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) (29). This, combined with early studies on co-culturing ASCs with biological scaffolds, has hopeful implications for the future implied by stimulation of neoangiogenesis.

Patient selection for ASC harvest: are there patient factors that improve ASC yield from aspirated fat?

Recent studies have investigated the impact patient factors, such as age and body mass index (BMI), have on numbers and viability of ASCs isolated from lipoaspirate. Van Harmelen investigated patients undergoing

breast reduction; this revealed that BMI correlates positively with age, mature fat cell size and total number of ASCs/fat cells per body. However, BMI correlated negatively with number of adipocytes and stromal cells per gram of adipose tissue, and with differentiation capacity. He found no relationship between BMI and ratio of other stromal cells to adipocytes (67). Padoin *et al.* found no link between BMI and age, and ASC yield (68). Another study focusing on age and donor sites for fat harvest found higher lipolytic activity in the younger population, with associated increase in rates of pre-adipocyte proliferation and differentiation, and lower levels of apoptosis (69).

Techniques of fat harvest and their implications for ASC retrieval

The major determinant of the ideal donor site is, of course, patient dependent (Fig. 1). Positioning of the patient during surgery may also contribute to a particular site being chosen in preference over another. A 2002 study by Rohrich *et al.* (31), has in the most part settled speculation about effect of donor site on fat cell viability. It found no statistical differences in adipocyte viability among abdominal fat, thigh fat, flank fat or knee fat.

Figure 1. Anatomical regions most commonly associated with liposuction (<http://www.aestheticsurgery.ie/images/liposuction1.jpg>).

These findings, obtained on untreated specimens, were echoed by those in centrifuged samples of the same donor sites. A more recent paper on adipocyte viability has agreed with these findings (27). Schipper's study, however, found ASCs in superficial abdominal fat (superficial to Scarpa's fascia) to be the most resistant to apoptosis, and therefore a superior fat donor site (69). A further study concurred that the abdomen is the optimum harvest site, citing medial thigh and knee to have the poorest ASC viability levels (68). A Dutch study by Jurgens *et al.* in 2008 analysed the relationship between fat harvest site and yield of stem cells, looking in particular into differentiation into chondrogenic and osteogenic lineages. They found a significant difference in ASC frequency, but not in absolute number of cells, between fat from the abdomen and thighs; differentiation capacity was unaffected. It was therefore concluded that the abdomen might be preferable for purposes of fat harvest for stem cell-based therapies (30).

Anaesthesia and the use of tumescence

Free fat transfer is usually performed under general anaesthetic with *in situ* infiltration of local anaesthetic fluid with or without adrenaline. Smaller volume fat transfers, particularly to the face, can be performed under local anaesthesia with sedation and/or analgesia.

Local anaesthetic infiltration directly into the donor site (usually the abdomen) is commonly a combination of lidocaine and adrenaline, occasionally with addition of bicarbonate. Volumes infused into the donor area have been under much scrutiny. The tumescent technique, so called due to tightness of the donor area that results from large amounts of subcutaneously infused fluid, is described as use of up to four times the volume of expected aspirate in local infiltration solution. It has been hypothesized that resulting distension of tissues and compression of blood vessels provides better haemostasis. This is of course augmented further by vasoconstrictive action of adrenaline.

Moore *et al.* have shown no difference in adipocyte viability after use of lignocaine over the long term; while adipocyte glucose transport was impeded for the duration of action of the lignocaine, it appeared to wear off with no lasting ill-effects (60).

Despite having a small sample size of eight patients, a study that compared effects of adrenaline concentration on adipocyte viability, has shown conclusively no statistical difference between groups of no adrenaline, and concentrations of 1:100 000, 1:200 000 and 1:400 000 (32), thus the overwhelming majority of fat transfer cases use adrenaline in its local anaesthetic infiltration of the donor site.

Figure 2. Technique of liposuction (<http://www.aestheticsurgery.ie/images/liposuction1.jpg>).

Harvest methods

Techniques for harvest of fat grafts affect not only viability of adipose-derived mesenchymal stem cells (Fig. 2), but crucially also their level of adhesiveness to key adhesion proteins (59). This in turn will influence our approaches to developing biotechnologies that are compatible with and complement the properties of ASCs. The majority of more recent studies carried out to compare liposuction harvest with syringe-aspirated [Coleman (56)] or excised fat, have revealed equivalent damage to pre-adipocytes (60–62) irrespective of harvest method used. It is generally accepted that a larger cannula diameter at harvest correlates with improved cell viability (38,44) (Table 1).

ASC isolation and purification techniques

The most prolific technique described for free fat transfer is Coleman's recommended method. He described centrifugation, in the clinical setting, at an optimum 3000 rpm for 3 min (56) (this study did not specify the more accurately used *g* units, or centrifugation equipment utilised, making comparison to other studies liable to error). This variably translates into G-force units, depending on apparatus used. This results in separation of harvested fat into three layers: infranatant (lowest layer composed of blood, tissue fluid and local anaesthetic), middle portion (primarily fatty tissue), and supranatant (upper layer, least dense and consisting of lipids). Infranatant components can be ejected from the base of the syringe, while supranatant can be poured off and soaked up using absorbent materials. While this is an extremely practical technique in an

operating theatre, even with minimal gadgetry, it may not produce the best fraction of adipose-derived stem cells possible.

Rodbell was the first to realize the importance of optimal purification of these cells in the 1960s, and started to develop purification techniques in a rat model (64). Many authors have now experimented with the aim of discovering the most efficient way to preserve cell viability and remove unwanted fractions (31,32,46,50–55). Dickens' study on characterization of mesenchymal progenitor cells from processed lipoaspirates (PLAs) revealed that while gentle centrifugation produced highest cell viability, long periods of centrifugation led to isolation of the most proliferative PLA cell type (65). Conde-Green has recently compared lipoaspirate from 20 patients, processed by decantation, washing and centrifugation. Histological analysis and flow cytometry have shown that while mesenchymal stem cell concentration was greater in washed lipoaspirates, pellets contained at the bottom of the centrifuged samples contained the highest concentration of stem cells (66) (Table 2).

Effect of cryopreservation on ASC survival

Many studies have investigated cryopreservation of ASCs as this could render the procedure of free fat transfer multiply more efficient, providing the possibility of a single episode of graft harvest, out of which multiple treatments could be born. It would appear that the majority of *in vitro* studies agree that cryopreservation of adipocytes in liquid nitrogen, preferably using a set cooling and rewarming protocol, is the least damaging to cell viability (33,34).

Table 1. Fat harvest methods and their effect on ADSC viability

Source of fat	Sample size (No)	Harvest technique	Viability assay	Outcome: viability of ADSCs	Refs
Human	8 versus 8	Coleman technique versus standard liposuction	G3PDH/trypan blue cell count/ histology	Coleman superior. Histology similar	(41)
Human	15 versus 9	Aspirated versus intact excised fat	G3PDH/histology/SVF culture	Capillaries damaged in aspirated fat 30% adipocytes ruptured in aspirated versus 5% excised fat ADSCs in aspirated tissue = 50% fewer than those of excised tissue	(42)
Human	6 versus 6	Coleman versus 680 mmHg vacuum aspiration	Histology/ microscopy and staining	Aspirated: less viability, more apoptosis	(43)
Human	6	2 mm versus 3 mm versus 4 mm harvest cannulae	Trypan blue cell count	Bigger cannula diameter → better viability rates	(44)
Human	10	2 mm versus 4 mm versus 6 mm cannulae	Trypan blue cell count	Bigger cannula diameter → better viability	(38)
Rat	5	Aspiration 10 cc syringe versus liposuction	Cell counts/MTT assay/ G3PDH/Oil Red O stain	Viability and plasticity better in aspirated graft	(45)
Murine inguinal	–	Aspiration 10 cc syringe versus excision		Viability best in excised fat grafts	(46)
Human	3	10 cc syringe versus aspirator	XTT assay	No difference in viability	(47)

This has been tested with a potential freezing timeframe of 6 months, with statistically no fewer viable adipocytes. These results have been replicated in an *in vivo* study (in mice), which revealed that grafts frozen in liquid nitrogen and stored at -35°C were of similar viability and histology to fresh tissue, whereas other methods of cryopreservation such as dry-freezing and immersion in glycerol resulted in marked adipocyte damage (35). A further Japanese study, *in vitro* (36), used electron microscopy, G3PD activity and cell surface marker expression to assess cell viability post-cryopreservation at various temperatures, demonstrated that while aspirated fat could be stored or transported overnight if preserved at 4°C , without adipose-derived stem cell yield loss or changes in biological properties, fat cryopreserved at -80°C for 30 days resulted in adipose-derived stem cell yield that was significantly less than that obtained from freshly aspirated fat. The most recently published data (37) examine use of a cryoprotective agent, dimethyl sulphoxide (DMSO), to increase adipose-derived stem cell yield, post-thawing. This indeed shows that while samples frozen with a cryoprotective agent had a lower ASC yield than that of fresh tissue, it was markedly better than samples processed without such an agent. Furthermore, when incorporated into an *in vivo* model, resulting fat grafts had greater weight and volume, as well as histological integrity (Table 3).

ASC isolation and culture

Isolation of a 'SVF', containing high concentration of pre-adipocytes, has emerged as the key to improving fat

graft take clinically, as well as to increasing pluripotent cell yield for differentiation into endothelial, osteogenic, chondrogenic and neural lineages. Stem cell yield of SVF of adipose tissue approaches 2%; a very significant concentration compared to 0.002% that has been quoted for bone marrow-derived mesenchymal stem cells (5,71). It has been found that pre-adipocytes tend to survive and proliferate better in culture media (72), and a variety of cell culture techniques and media have been compared.

To isolate ASCs, fresh lipoaspirates are routinely washed in phosphate-buffered saline, and digested in culture media using collagenase. Tissue fragments are further minced and incubated at 37°C , and neutralized in an alpha- or Dulbecco's modified Eagle's medium containing foetal bovine serum. Pipetting allows further separation of fat particles. Repeating the process of centrifugation then enables isolation of the ASC-rich pellet. This can be cultured and expanded in media, or cryopreserved (73) (Fig. 3).

As this field progresses, new techniques are being developed to enhance ASC yield further, from lipoaspirates. Rada *et al.* describe a standard novel method for isolation of ASCs through exposure to immuno-magnetic beads coated with antibodies, which separate out ASCs and identify further subpopulations of stem cells in adipose tissue (82).

Adipose-derived stem cells have proven problematic to identify in culture, and studies have been carried out to point to particular cell markers, which may make them easier to recognize. Lin *et al.*, in observing behaviour of ASCs in culture, likened them to vascular and endothelial

Table 2. Effect of centrifugation speed and time on ADSC survival.¹

Subjects	Group 1	Group 2	Group 3	Time	Assay	Viability	Refs
8 human samples	1500 rpm	3000 rpm	5000 rpm	1 min 3 min 5 min 2 min	Trypan blue cell count	3000 rpm 3 min recommended	(32)
5 patients, 8 samples each	500 g	Unspun	–	–	XTT assay	No significant difference	(31)
Human samples grafted into mice	300 g	Rolled on Telfa gauze × 2	Unprocessed	3 min × 2	FACS, G3PDH VEGF-ELISA	Centrifuge better than unprocessed; gauze best Graft survival at 2/52; gauze better than centrifuge, better than unprocessed	(48)
Human samples injected into mice	300 g, separated into highest and lowest density	HD	LD	3 min 2,4,6/52	FACS, ELISA, G3PDH Volume/weight	HD had more progenitor cells, more VEGF and more SDF LD had more G3PDH HD grafts better survival	(49)
20 human samples	50 g	–	–	2 min 4 min 6 min 8 min 5 min × 2	Trypan blue cell count	No increase in yield with >2 min centrifugation Bottom layer–highest number of viable adipocytes	(50)
Human samples from 1 patient; injected into mice	1500 rpm	Cotton towel → spoon → syringe	–	–	Graft weight/volume	No statistical difference in graft survival	(51)
Human samples	1000 rpm	5000 rpm	–	5 min	MTT assay	No significant difference in ADSCs	(52)
Human samples, injected into 57 mice	500 g versus control	Washing-Ringer's (no centrifuge)	Washing + centrifugation	2 min	XTT assay	Better viability with less handling	(47)
Mouse inguinal fat pads	500 rpm	–	–	3 min	Trypan blue cell count	1000 rpm for 3 min best viability	(46)
		1000 rpm	–	1,2,3,5,10 min 3 min			
	1500 rpm	–	1500 rpm				
	2000 rpm	–	2000 rpm				
Human fat 8 patients; injected into mice	0 g 400 g 3000 g	– 800 g 4200 g	– 1200 g	– 3 min 3 min	Graft weight/volume at 4/52	>3000 g causes adipocyte damage Centrifugation at 1200 g for 3 min enhanced graft take	(53)
24 samples from 22 patients	Sedimentation	3000 rpm for 3 min	Centrifugation and washing	3 min	Histology	Sedimentation increases viability. More handling = less survival	(54)
14 patients	Non-centrifuged	3600 rpm	–	3 min	Clinical evaluation	Better results with centrifuged fat	(55)

¹ All centrifugation values given in g units unless studies reported in rpm and omit to specify centrifugation equipment used.

Table 3. Effect of cryopreservation on ADSC survival

Subjects	Methods	Temp (°C)	Time	CPA	Assays	ASC yield	Refs
Human	Slow cooling/fast warming	-196	4/12	DMSO/trehalose	Weight, volume, histology	Best fresh but cryoprotective effect helps	(37)
Human	Liq N ₂ , cooling 1 °C/15 min, fast rewarming	-196	20 min	DMSO/trehalose	Flow cytometry/G3PDH/Histology	Best fresh. Better with cryoprotectant if frozen	(33)
Human	Freezing medium; slow cooling, water bath 37 °C to rewarm	-80	1/12	-	Electron micros copy/G3PDH/Flow cytometry	4 °C 24 h – no compromise in viability. Freezing decreases yield	(36)
Human; ultrasound pre-treatment	1 °C per min cooling; rapid rewarming	-196	6/12	Cell Banker 1 (containing foetal bovine serum)	Doubling time in cell culture; differentiation assays; flow cytometry	Proliferation unaffected by freezing. Differentiation unaffected but larger variability	(34)
Mice fat donor animal	Dry freezing; glycerol; Liq N ₂ ; thawing at room temp for 1 h	-35	6/12	-	MTT reduction test; histology	Liq N ₂ sample adipocytes unaffected; others – decreased viability	(35)
Human	Storage at three different temps; thawing 1 h at room temp	+4 -20 -80	2/52	-	Supravital dye/haemocytometry	+4 °C – statistically no difference compared with fresh -20 °C and -80 °C – significant decrease in viable adipocytes	(38)
Human	Commercial freezer	-20 -80	0, 2, 7, 14 & 30 days	Various combinations	MTT/XTT assay G3PDH	Freezing – loss of 92.7% metabolic activity; CPA preserves 54% viability.	(39)
Human	Simple freezing versus – 1 °C/min + CPA	-20	Min-h	Group 2 only	Staining/culture	Group 1 – very low viability. Group 2 – preserved	(40)
Human Injected into mice	Slow cooling by 1–2 °C to -30 °C, -196 °C with Liq N ₂ . Fast rewarm in 40 °C water bath	-196	Min-h 4/12	DMSA/trehalose Untreated versus CPA	Adipocyte counts/histology Graft weight/volume	Fresh samples best viability, CPA samples good preservation Improved graft weight/volume with CPA	(23)
6 human lipospiates	Liq N ₂ versus control culture	-	-	Me ₂ SO	Flow cytometry/DNA assay	Cryopreservation has no adverse effect on phenotype, proliferation or differentiation	(70)

Figure 3. Procedure for extraction of ADSCs from aspirated adipose tissue [adapted from (73)].

Figure 4. Alizarin Red staining of adipocytes/ASCs (http://guldberglab.ibb.gatech.edu/images/04_hADSC_miner_wtext.jpg).

cells, and pinpointed markers CD34+CD31-CD104b-SMA- in this differentiation (57,96).

Multiple types of culture media (Fig. 4) have been trialled to observe behaviour of ASCs. Balwierz' study has shown that presence of serum and absence of adipogenic factors promotes endothelial differentiation of ASCs of a stromal vascular fraction; their actions in combination with human umbilical vein endothelial cells (HUVEC) resulted in formation of vascular networks (86). Mischen *et al.* tested metabolism, proliferation and differentiation of ASCs under 16 media conditions: differing glucose concentrations, glutamine and hypoxic medium. Glucose, lactate and pyruvate content of media were assessed to ascertain activity of ASCs. While active in all these conditions, ASCs increased glucose consumption and lactate production in a hypoxic environment, and only exhibited osteogenic differentiation in physiologically normal to high glucose and

oxygen conditions. With high oxygen content, more proliferation and cell death were detected (80).

Platelet-rich plasma has long been a hot topic for potential of increasing ASC survival in fat grafts. Por *et al.* compared fat grafts of human lipoaspirate injected into 24 mice, with platelet-rich plasma (PRP), or saline as control. At 16 weeks, graft weights and volumes were measured; no difference in graft survival was found (74). This is in contrast to other studies, which show increased ASC proliferation with PRP (75,76), and improved graft viability (77).

While current approaches clearly achieve substantial results in isolation and culture of ASCs, culture media used carry with them risks of infection stemming from their serum derivation. New strategies are in place for accomplishing similar outcomes with use of serum- and xeno-free media (81).

Cell-assisted lipotransfer

The autologous fat transfer technique of cell-assisted lipotransfer describes addition of purified SVF to PLA. This usually follows routine liposuction, whereby half the lipoaspirate is centrifuged according to Coleman's method, while the other half is washed, enzymatically digested, filtered and spun down to the ASC-rich pellet fraction. The latter is then mixed with the former to make up a 'turbo-charged' fat graft (83,84). Most recently, this has been supported by findings from Cytori (2010), in their *in vivo* murine study. ASCs increase graft retention by a factor of two, and improve overall quality of the grafts. It is thought that increased angiogenesis, and resulting capillary density, appear to promote neovascularization and adipocyte differentiation, and prevent apoptosis, through expression of VEGF and IGF-1 (85).

Use of biomaterials in ASC culture

Numerous technologies have been piloted as scaffolds for anchoring ASC-rich fat grafts for soft tissue reconstruction. Scaffolds can serve both as mechanical stabilization for cells, and to promote differentiation and eventually tissue organization. Injectable scaffolds, such as poly (lactic-co-glycolic acid) spheres, provide the advantage of avoidance of surgery, ability to self-fill the required defect and potential for alteration *via* injection of various factors, while forming adipose tissue *in vivo* in 8 weeks (69).

Collagen is a basic, commonly used, biodegradable, tissue-derived scaffold that permits co-culture of multiple types of cells, often in the form of a hydrogel, mimicking real tissue. It can remain *in situ* for the length of time necessary for graft cells to integrate fully into their new environment, or for up to years if required. Structure of the scaffold is subject to alteration, and varying densities produce different results. Additional growth factors can be superimposed into the scaffold to augment or change the desired effect, or alter properties of adhesion (78, 79, 87). Collagenous microbeads allow *ex vivo* proliferation and differentiation on particles that are small enough to be injectable. A study by Rubin *et al.* has shown that collagenous microbeads are a favourable environment for proliferation and differentiation of ASCs into adipogenic and osteogenic lineages when concurrently exposed to stimulatory media (88).

Hyaluronic acid gel has been shown to promote cell proliferation and population expansion of ASCs as well as controls; in an *in vivo* murine model, this has translated into stable filling volumes and a solid fibrovascular network (89). Other materials that have been deemed suitable

as scaffolds for stem cells include a spectrum of polymers and esters—perfluoroelastomers seeded with ASCs (91) (these are non-biodegradable), chitoooligosaccharide/chitosan compounds (92) and fibrin glue (93). Materials such as placental decellular matrix and cross-linked hyaluronan scaffolds have also been shown to promote cell proliferation, viability and differentiation capacity (66,88).

Non-biodegradable materials that become well incorporated into the new tissue present the challenge of not allowing optimum cell adherence and proliferation (as they tend to be inert compounds). Vallee *et al.* have proposed a novel technique for creation of a fat scaffold, starting with standard isolation of ASCs. After 21–28 days culture, ascorbic acid stimulation and induction of adipogenesis with rosiglitazone, with concurrent secretion of extracellular matrix, malleable fat 'sheets' were assembled. Layering these produced relatively bulky 'filler' fat sheets suitable for reconstruction of soft tissue defects. Even though an *in vitro* study, this is clearly a significant development in creation of autologous soft tissue scaffolds (63). A Korean study has also shown Alloderm (a regenerative tissue matrix, based on cadaveric skin) to be a suitable matrix for the integration of ASCs into a recipient site (90).

Additional factors in induction of ASC proliferation and differentiation

As living cells, ASCs secrete a number of factors and genes (102) which have been studied to determine their effects on proliferation and differentiation. Rehman isolated ASCs, which were then characterized by flow cytometry, to observe production of VEGF, HGF and TGF-beta. Levels of VEGF appeared to increase with hypoxia, therefore prompting an increase in angiogenesis (94,95). Lu studied VEGF specifically in a murine model, and found fat graft tissue volumes to be improved, with enhanced capillary networks on histological examination (98). HGF is likewise linked with increased angiogenesis (97) and TGF-beta has been observed to be crucial for increasing proliferative capacity of ASCs (96). Another study by Mehlhorn *et al.* showed that differentiation of ASCs into chondrocytes was maximized by simultaneous treatment with bone morphogenic protein 2 and TGF-beta1 (58).

Fibroblast growth factor (FGF) also plays a vital role in neovascularization (99–101). Eppley *et al.* found nearly complete graft take, at 6 months (100) in a rat model ($n = 20$), with implanted ASCs, supplemented by beta-FGF. Sajjadian *et al.* showed beta-FGF to be a stimulatory signal, promoting pre-adipocyte migration and local neovascularization. Insulin and IGF-1 showed evidence of

being involved in subsequent adipocytic differentiation. Sung studied both effects of beta-FGF and insulin (not in combination) on fat grafts in 24 rabbits. Both of these showed increase in numbers of mature adipocytes within the grafts, and lessened the likelihood of cyst formation and fibrosis (101). Preceding experiments, however, found no significant difference in pre-adipocyte viability with the use of insulin (51). Raposio showed considerable increase in cell population growth of human pre-adipocytes by use of polydeoxyribonucleotide (PDRN) (71). Other factors, such as PDGF-AB and GCSF (97), may also be instrumental in proliferation of ASCs and angiogenesis, respectively.

Lee *et al.* examined proliferation and differentiation ability of ASCs when treated with histone deacetylase (HDAC) inhibitors, which are of relevance to cancer therapeutics. Cell-flattening properties of these demonstrated effectively that HDAC is necessary for ASC cell renewal and proliferation (106).

Strategies for the future

While many trends have been observed in relationships between ASCs and biotechnologies, much work is anticipated in product development, incorporating newer biomaterials and nanotechnologies, which can be put into practice clinically to enhance our ability for filling soft tissue defects for surgical reconstruction. Alongside these advances, a variety of factors, mediators and genes have been implicated in manipulation of proliferation and adipogenic differentiation of ASCs, and much research will lie in defining these interactions (28,106–108).

There has been interest in exploring the potential of pluripotent ASCs to trigger relapse in patients with a previous history of malignancy at an injection site, or recurrence in a previous radiotherapy-treated field. While there is little evidence to provoke significant concern as yet, definitive studies are needed to assess these risks.

De novo adipogenesis is another avenue to be more fully explored with a view to increasing our reconstructive options. This requires spontaneous origination of pre-adipocytes, with subsequent angiogenesis and vascularization, followed finally by differentiation into mature adipose tissue. This is currently under trial in *in vitro* and *in vivo* animal studies (103–105).

Conclusion

While clearly much work has gone into researching into the various methods of obtaining and purifying fat tissue to invoke optimum benefit in terms of proliferation and differentiation capacities, there is little resonance

between the studies, and hard evidence is yet to be arrived at. As a rapidly expanding field, novel biotechnologies are constantly being put forward to solve the plastic surgeon's greatest dilemma – how to achieve seamless defect reconstruction.

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