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Identifying polymers that support the growth and differentiation of adipose derived pericytes for use in auricular reconstruction.

Christopher C. West

Submitted for the degree

Doctor of Philosophy

University of Edinburgh

2017
Declaration

This thesis is entirely written by myself. It contains only my own work, and includes nothing that is the outcome of work done in collaboration, except where specifically indicated in the text.

The data presented in this text has not been submitted for any other degree or professional qualification.

Christopher C. West
Abstract

In the United Kingdom 1 in 6 - 8000 children are born missing one or both of their ears. The surgical technique most commonly used to reconstruct ears requires surgeons to remove ribs from the patient, and the cartilage from the ribs is used to carve a new ear. This procedure involves many risks including significant pain, punctured lung and chest deformity. Therefore the ability to ‘grow’ an ear would be a major advancement.

Stem cells show huge promise in tissue engineering and regenerative medicine. Approved stem cell technology must be evaluated with regards to safety, purity, identity, potency and efficacy prior to biologic licensing and clinical use. Therefore, access to ethically sourced tissue for research is fundamental to the successful delivery of novel therapies. Adipose tissue provides an abundant and accessible source of stem cells for clinical translation. Within the first section of this thesis, the perceptions and attitudes of patients towards the donation and use of adipose tissue for research are sought. Based on this information, a tissue bank with all appropriate ethical approval to collect, process, store and distribute adipose tissue and adipose derived stem cells is established.

The second part of this thesis demonstrates the specific identity, location and frequency of stem cells within adipose tissue; revealing them to reside in a perivascular niche. Using this data, protocols to rapidly purify stem cells from
adipose tissue using Fluorescence Activated Cell Sorting are developed. The frequency of cells, and both the patient and procedure based variables that can affect this yield are also examined.

The final section of this thesis uses a high-throughput microarray platform to screen thousands of polymers to identify potential substrates that can support the attachment, stable proliferation and subsequent differentiation of stem cells purified from adipose tissue. From the initial screen, 5 distinct polymers have been identified, characterised and their effects on the stem cells examined and quantified.

Combined together, these elements provide significant advances in our understanding, and the basis for on going research to deliver a tissue engineered ear for use in human ear reconstruction.
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Finally, I would like to thank my parents whose love and support is endless. Thank you for encouraging me to pursue my dreams, and for supporting me every step of the way.
Dedication

To Liz, Abigail and our future.

There is no greater pleasure than the time we spend together.
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List of Abbreviations

ASC, Adipose derived stem/stromal cell
αSMA, Alpha smooth muscle actin
BMI, Body Mass Index
BSA, Bovine serum albumin
CD, Cluster of differentiation
CFU, Colony forming unit
DMB, Dimethyl blue
DMEM, Dulbecco’s modified eagle medium
ES, Embryonic stem cell
ESPRAS, European Society of Plastic, Reconstructive and Aesthetic Surgeons
EURAPS, European Association of Plastic Surgeons
FACS, Fluorescence activated cell sorting
FCS, Foetal calf serum
GAG, Glycosaminoglycan
GMC, General Medical Council
GMP, Good manufacturing practice or Good medical practice
GVHD, Graft versus host disease
HCS, High-resolution high-content screening
hESC, Human embryonic stem cell
HTA, Human Tissue Act or Human Tissue Authority
ICC, Immunocytochemistry
IHC, Immunohistochemistry
IFATS, International Federation for Adipose Therapeutics and Science

IHD, Ischaemic Heart Disease

iPS, Induced pluripotent stem cell

IRAS, Integrated research application system

MRC, Medical Research Council

MSC, Mesenchymal stem cell

MTA, Material transfer agreement

NHS, National Health Service

NMP, N-Methyl-2-pyrrolidone

OA, Osteoarthritis

PA, Polyacrylate

PBS, Phosphate Buffered Saline

PCL, Polycaprolactone

PDGFRβ, Platelet derived growth factor receptor beta

PFA, Paraformaldehyde

PGA, Polyglycolic Acid

PLA, Polylactic Acid

POSS-PCU, Polyhedral Oligomeric Silsesquioxanes - Poly(carbonate-urea)urethane

PSC, Perivascular stem cell

PU, Polyurethane

RA, Rheumatoid Arthritis

REC, Research Ethics Committee

RTB, Research Tissue Bank

SARS, Society of Academic Research and Surgery
SGM, Standard Growth Media
SVF, Stromal vascular fraction
THF, Tetrahydrofuran
UCLA, University of California at Los Angeles
UK, United Kingdom
UoE, University of Edinburgh
1 Introduction

Parts of this chapter have previously been published in:

1. **CC West**


2. Murray IR, **West CC**, Hardy WR, James AW, Park TS, Nguyen A, Tawonsawatrak T, Lazzari L, Soo C, Peault B.


   Natural History of Mesenchymal Stem Cells, from Vessel Walls to Culture Vessels


   *Pharmacology and Therapeutics.* 2016 Aug 7

   Pericytes for the treatment of orthopaedic conditions
1.1 Regenerative medicine and plastic surgery

Regenerative medicine is a rapidly expanding field of research, with many strategies currently being translated toward the clinic (Giordano et al. 2007). Regenerative medicine is defined as;

“process of replacing or regenerating human cells, tissues or organs to restore or establish normal function” (Mason & Dunnill 2008).

This definition is very similar to that of plastic surgery that aims to achieve;

“restoration of form and function”.

Throughout the history of plastic and reconstructive surgery, the primary challenge facing surgeons has been finding suitable donor tissue that can reconstruct the defect without causing significant additional disfigurement and disability. Common defects encountered that require reconstruction include multiple composite tissues such as skin, adipose tissue, muscle, bone, nerve and cartilage. Whilst the potential application of regenerative strategies to plastic surgery is huge, the focus of this thesis will be its application to ear reconstruction surgery.

Ear reconstruction is widely regarded as one of the most technically demanding reconstructive challenges. For this reason surgery is usually only performed by small numbers of surgeons who are experts in this particular
type of procedure. The Scottish Ear Reconstruction Service ([http://www.nsd.scot.nhs.uk/services/specserv/autearrecon.html](http://www.nsd.scot.nhs.uk/services/specserv/autearrecon.html)) is run by Mr Ken Stewart, a consultant plastic surgeon based at the Royal Hospital for Sick Children in Edinburgh. Approximately 40 new patients are treated each year covering referrals from all of Scotland and other parts of the UK.

### 1.2 The Ear

The external ear consists of the auricle or pinna, and the external ear canal. It is a complex piece of elastic cartilage covered in the most part by hairless skin, and there is wide variation in its size, shape and position (Tolleth 1978). The primary function of the pinna is to channel sound waves into the ear canal and reflect them efficiently on to the tympanic membrane. It also serves to block out unwanted noise from other directions. People who lack a pinna but have a patent ear canal report loss of specific noise volume, with an increase in general interference from unwanted background noise. Ears have also developed a number of secondary functions such as holding reading glasses, sunglasses and headphones. In addition to this, they are important paired aesthetic units that contribute to the normal appearance of the face. Amputation and mutilation of the ears has been used for centuries in different cultures as a form of humiliation, retaliation and punishment for a multitude of crimes (Cane 1885).
1.2.1 Anatomy

The auricle is a complex 3-dimensional structure whose delicate and intricate architecture is a result of the interlocking curves and tensile forces of the fibroelastic cartilage underlying the skin. The auricle is comprised of 3 major units that are the helix-antihelix, the concha and the lobule. Within these units there are many other smaller subunits which each contribute to the overall appearance (Figure 1.1). Minimal disruption to any of these can lead to significant alterations in the overall appearance of the ear.

Figure 1.1: Topographical anatomy of the ear
The auricle begins to develop between the 3\textsuperscript{rd} and 6\textsuperscript{th} weeks, with definitive features evident by the ninth week, and is fully formed by the fourth month (Figure 1.2). It begins as 6 precartilaginous swellings (hillocks) of mesodermal tissue that arise on the first (mandibular) and second (hyoid) branchial arches with the pharyngeal groove lying between them. These grooves and arches represent what were the primordial ‘gill slits’ of a fish. The auricle develops around the external auditory meatus that forms from the first branchial cleft that begins to canalise at 28 weeks.

In the early classic description of ear embryology by His, the anterior 3 swellings that arise on the first arch formed the tragus, the helical root and the superior helix and the posterior 3 swellings from the second arch form the antihelix, antitragus and lobule. However, in later descriptions it was suggested that these hillocks are just incidental and represent early foci of mesenchymal condensation and proliferation of the underlying branchial arches that subsequently equalize with the surrounding tissue (Washington 1922).
Figure 1.2: The embryological development of the ear. A.) 6 early swellings (hillocks) present from the 3\textsuperscript{rd} gestational week. B.) Fusion of the hillocks is apparent at 6 weeks. C.) The appearance of the adult ear.

The ear begins to develop in an infero-medial position below the mandible, and by the 20th week migrates to a postero-superior position overlying the temporal bone. This rapid sequence of events that results in the migration of the pinna can be disrupted by a range of genetic and environmental factors, with a variety of resulting abnormalities. Therefore patients who present with premature arrest or other congenital auricular abnormalities frequently have low (caudal) and anteriorly set ears (Pham et al. 2014). As the migrating pinna crosses multiple structures, it is not surprising that over 50\% of children with severe ear anomalies also have other associated craniofacial anomalies.

Sensation to the ear is supplied by both cranial and extra cranial nerves that have a varied distribution. The lesser occipital and great auricular nerves supply the posterior surface of the auricle and the lobule. The lesser occipital
being the dominant nerve supplying the superior ear and mastoid fossa. Injury to the great auricular nerve is common in surgical approaches around the infero-posterior border of the ear (Pantaloni & Sullivan 2000). The anterior surface of the auricle and the tragus are supplied by the auriculotemporal branches of the 3rd (mandibular) division of the trigeminal nerve. Sensation to the external auditory meatus is from the auricular branch of the vagus nerve (Arnold's nerve) (Beahm & Walton 2002).

The blood supply of the auricle is by 2 distinct but interconnecting networks both derived from the external carotid artery. An understanding of the blood supply to the auricle and surrounding soft tissues is of paramount importance in ear reconstruction surgery to prevent necrosis when dissecting and transposing structures.

Original descriptions of the lymphatic system suggest that the drainage reflects the embryonic development with the concha and meatus draining to parotid and infraclavicular nodes, and the external meatus and superior auricle drain to mastoid and superior cervical nodes. Subsequent lymphoscintigraphy studies have demonstrated that patterns are frequently more complex and unpredictable than originally thought.

There are 3 extrinsic muscles of the ear which are the anterior, superior and posterior auricularis muscles. In addition there are 7 intrinsic muscles which are mainly vestigial and they are the transverse and oblique muscles,
tragicus, antitragicus, helicis major and helicis minor (Beahm & Walton 2002).

1.3 Auricular anomalies

The exact incidence of auricular anomalies is hard to estimate as they encompass a huge spectrum of malformations, and the less severe are often under reported. The most severe types of malformation such as microtia or anotia are rare and occur between 0.8 and 8.3 per 10,000 live births. Incidence of the least severe auricular malformations may be as high as 55% (Porter & Tan 2005). Many different classification systems have been suggested to address these anomalies however no one particular system appears to satisfy the issue. In their broadest form, congenital anomalies can be categorized in to either malformational or deformational. The former are due to aberrations in the normal embryological development of the ear, whilst the latter are due to either in utero or ex utero deformational forces applied to normal ears (Tan et al. 2003). The two may occur concurrently as malformed ears are more likely to encounter deformational force (Porter & Tan 2005). Tanzer suggested a classification for auricular defects based on the severity of the defect (Tanzer 1959).

- Class I: Anotia
- Class II: Complete hypoplasia
  - II A: with atresia of the auditory canal
  - II B: without atresia of the auditory canal
• Class III: Hypoplasia of the middle third of the auricle
• Class IV: Hypoplasia of the superior third of the auricle
  o IV A: Constricted (cup or lop)
  o IV B: Cryptotia
  o IV C: Hypoplasia of entire superior third
• Class V: Prominent ears

A widely accepted and used classification scheme which links the severity of the defect to the degree of reconstruction required is that proposed by Weerda (Weerda 1988);
• 1st degree dysplasia: Well formed auricle with clear but minor deformity. No additional tissue required for reconstruction
• 2nd degree dysplasia: Some recognizable features within a rudimentary and moderately disfigured pinna. Partial reconstruction with skin and/or cartilage is required.
• 3rd degree dysplasia: Severe disfigurement of the pinna with no recognizable features requiring total reconstruction.

1.4 Microtia

Microtia is a rare developmental malformation of the auricle with an estimated prevalence between 0.8 and 4.2 per 10,000 live births (Alasti & Van Camp 2009). It has a relatively low prevalence in European and Blacks compared to Asian and Hispanic races, with the highest recorded prevalence seen amongst the Japanese and Navajo Indians (Porter & Tan 2005).
Unilateral cases represent 90% of microtia cases, with the right ear twice as likely to be affected than the left ear. It is more frequent in males with a ratio of 2:1 (Alasti & Van Camp 2009). Half of patients who present with microtia also have other features of first and second branchial arch syndrome. This led to suggestions that isolated microtia is the mildest form of hemifacial (craniofacial) microsomia. It is also associated with syndromes such as Treacher-Collins and Goldenhaar (Figure 1.3).

**Figure 1.3: Microtia and associated syndromes.** A.) Bilateral microtia in a patient with Treacher-Collins syndrome, with bone anchored hearing aid on the right side. B.) Unilateral microtia in a patient with craniofacial microsomia.
1.4.1 Classification of microtia

Although general classifications of auricular anomalies have previously been described (see above), the most widely accepted classification specific to microtia is that of Nagata. This classification is particularly useful as the classification also correlates to the surgical procedure used to reconstruct it (Nagata 1994a; Nagata 1994b; Nagata 1994c).

Lobule type

These deformities are characterised by the presence of a malpositioned lobule and ear remnant. There is no concha, acoustic meatus or tragus. (Nagata 1994a)

Concha type

These patients have an ear remnant comprising of a malpositioned earlobe, concha (with or without acoustic meatus), tragus and antitragus. (Nagata 1994b)

Small concha type

Similar to conchal type, however these patients have a small indentation instead of a concha. (Nagata 1994c)

Anotia

Patients with no, or a very tiny ear remnant

Atypical microtia

Ear deformities that do not correlate with any of the above categories
Fig 1.4: Examples of common sub-types of microtia based on the classification system described by Nagata (Nagata 1994). A.) Lobular type characterised by the presence of a malpositioned lobule and ear remnant. There is no concha, acoustic meatus or tragus. B.) Conchal type comprising of a malpositioned earlobe, concha (with or without acoustic meatus), tragus and antitragus. C.) Anotia with no, or a very tiny ear remnant.

1.4.2 Treatment of microtia

Total ear reconstruction is a relatively new procedure, with most modern techniques being pioneered in the second part of the 20th century. There are a number of historical cases of partial ear reconstruction documented. According to The Susruta, an Indian text of ancient medicine, reconstruction of the earlobe was performed using a partial cheek flap in 900BC. Tagliacozzi, an Italian surgeon, described the use of a transferred flap from the arm to reconstruct an auricle in 1597. In the 18th century a case using a folded mastoid flap was described to reconstruct a traumatic defect (Converse 2014).
Total ear reconstruction for microtia was thought to be impossible, and resulted in the trial of a number of unsuccessful materials to create a new framework including bone, allografts and xenografts (Romo & Reitzen 2008). In the 1930s the experimental and clinical use of costal cartilage as an autologous graft paved the way for modern reconstructive techniques used in microtia (Pierce et al. 1947). Most modern techniques use a variety of materials to fabricate a framework that is placed under the skin to create an ear form. Although different surgeons have advocated the use of different materials, autologous costal cartilage is widely regarded as the gold standard (Beahm & Walton 2002).

1.4.2.1 Reconstruction using autologous cartilage
All of the modern versions of autologous cartilage reconstruction are adaptations of the pioneering work of Tanzer who described and evaluated his technique of creating a carved cartilage framework (Tanzer 1959; Tanzer 1971; Tanzer 1978). Notable variations have been made by surgeons including Brent (Brent 1980a; Brent 1980b) and Nagata (Nagata 1993; Nagata 1994a; Nagata 1994b; Nagata 1994c).

1.4.2.1.1 Tanzer technique
Tanzer's original description was a 6 stage procedure using contralateral costal cartilage and composite skin and cartilage grafts from the contralateral ear (Tanzer 1959).
• Stage 1: Transposition of the lobular remnant to its normal anatomical position.

• Stage 2: Performed when the swelling and induration from stage 1 has resolved. Framework is created by harvesting cartilage sub-perichondrally from the contralateral 6th-8th ribs. Ribs 6 and 7 are used to construct the base and antihelix, with the 8th rib being used to create the helical rim. The different pieces of the framework are secured together using fine gauge wire.

• Stage 3 and 4: Performed 4 months after stage 2. The framework is elevated from the side of the skull to give a more natural projection. Postauricular skin is advanced, and the retroauricular sulcus that is created is covered with a split thickness skin graft. This is done initially at the inferior aspect (Stage 3) followed 2 months later at the superior aspect (Stage 4), to preserve to blood supply to the ear.

• Stage 5: Performed 4 months after stage 4. Closure of the ear tunnel with a retroauricular flap with either a split, or full thickness graft.

• Stage 6: Performed 6 weeks after stage 5. The concha and tragus are reconstructed using composite skin and cartilage grafts from the contralateral ear.

Laterly Tanzer refined this technique by combining stages 1 and 2. However in cases where extensive mobilisation of the lobule was required, the original 6 stage description was advocated to minimize the risk of vascular compromise to the lobule (Tanzer 1978).
1.4.2.1.2 Brent technique

The technique developed by Brent is a 3 or 4 stage technique based on the technique of Tanzer, but where the sequence of reconstruction is varied along with other modifications.

• Stage 1: A framework is created from the contralateral ribs. The synchondrosis of the 6th and 7th ribs is used to form the base, and the floating 8th rib is used to create the helix. Clear non-absorbable sutures are used to join the various elements together. Brent also exaggerates the 3-dimensional features of the construct to overcome the effects of the thickened overlying skin pocket. This is augmented by the use of suction drains which have the added advantage of reducing the complication rate associated with pressure dressings (Brent 1992).

• Stage 2: Transposition of the lobule is performed several months after the first stage, and the perceived advantage to this change in order is to minimize the disruption to the vascular supply of the skin flaps from the dissection required to transpose the lobule. In some cases this is combined with the 3rd stage.

• Stage 3: The construct is elevated as in the Tanzer technique described previously.

• Stage 4: Brent uses the framework to reconstruct the tragus. At this stage he also exaggerates the conchal bowl by excavating it, and also performs any further symmetrizing procedures.
1.4.2.1.3 Nagata technique

Nagata’s original 2 stage technique was first described in 1993 (Nagata 1993), with subsequent revisions and refinements made by the author based on the different type of microtia encountered as described in his eponymous classification.

- Stage 1: Creation of a cartilage framework from the ipsilateral 6th – 9th ribs, with concurrent transposition of the lobule and tragal reconstruction.
- Stage 2: The construct is elevated 6 months following the initial stage with a crescenteric piece of costal cartilage placed behind the ear to increase projection. A combination of temporoparietal fascial flap and split thickness skin graft from the scalp, or advancement of the retroauricular skin and graft is used to create and resurface the postauricular sulcus.

1.4.2.1.4 Complications of autologous reconstruction

The complications and morbidity associated with autologous reconstruction, particularly those pertaining to the donor site, are not insignificant and have been the principle criticism of this type of reconstruction. Complications associated with the reconstruction site include scarring, infection, flap necrosis, skin loss, exposure of the framework and extrusion. Complications associated with the harvest of costal cartilage include scarring, significant
post-operative pain, chest wall deformity, pneumothorax and thoracic
deformities (Uppal, Sabbagh, Chana & Gault 2008a; Thomson et al. 1995).

Uppal et al published series of 26 patients undergoing costal cartilage
harvest for ear reconstruction (Uppal, Sabbagh, Chana & Gault 2008b). They
reported significant postoperative pain that peaked at day 7, and continued
for over 3 months in 12% of patients. Thomson et al (Thomson et al.
1995) followed up their series of 80 patients to evaluate the appearance of
chest scars, topography and contour deformities of the donor site. 14% of
patients considered their scars unacceptable, with increasing age correlating
to poorer scarring. 25% of patients had noticeable retrusion in chest wall
topography with 6% of patients having severe retrusion. Younger patients
were more likely to encounter severe retrusion. All patients demonstrated
some degree of alteration in their ribcage contour. Another study examining
chest wall deformities and thoracic scoliosis following costal cartilage
harvesting noted chest wall deformities in 50% of the 18 patients in their
study. They also observed that younger patients were more likely to develop
deformities compared to older patients. In addition, they noted thoracic
scoliosis in 25% of the patients in their study (Ohara et al. 1997).

In attempt to minimize the morbidity associated with the donor site harvest,
techniques have been described that aim to preserve the regenerative
capacity of the ribs (Yang et al. 2015; Fattah et al. 2010). Nagata advocates
the subperichondral elevation of cartilage which preserves a perichondral
pocket to which the excess cartilage can be returned. This method allows regeneration of histologically mature cartilage at 12 months that is of sufficient bulk and quality to be used in subsequent reconstruction (Kawanabe & Nagata 2006). Using this technique, improvements in the chest wall contour have been noted (Uppal, Sabbagh, Chana & Gault 2008a). Fattah et al analysed 38 patients, 23 who had reconstruction of the rib with the preservation of the perichondrium versus 15 who did not. In those patients who did not have reconstruction, all had a palpable defect, whereas those who did have reconstruction had no palpable defect (Fattah et al. 2010).

Extrusion of autologous costal cartilage frameworks leading to their removal has not been commonly reported in any of the large published series (Brent 1992; Brent 1980a; Tanzer 1978; Fukuda & Yamada 1978; Wray & Hoopes 1973). The largest single series of autologous ear reconstruction was published by Brent in 1992 (Brent 1992). In this series he reported significantly lower complication rates compared with others, and also when compared to his own earlier series. This reduction was attributed to the introduction of suction drains to replace the bolster sutures and pressure dressings previously used to coapt the skin to the underlying framework. In this series he reported an infection rate of 0.5% and skin loss rate of 0.8%, compared to his earlier series with rates of 33% (Brent 1980b).
1.4.2.2  Reconstruction using synthetic frameworks

Due to the complications associated with harvesting costal cartilage, the use of synthetic frameworks has been advocated by a number of groups. The majority of these frameworks are silicone based or polyethylene based eg. Medpor®. In addition to eliminating the need to harvest costal cartilage, synthetic implants eliminate the associated operator variability in the quality of frameworks carved from costal cartilage, and the potential for loss of contours which has been described with resorbtion of cartilage frameworks (Tanzer 1978). Conversely, there is no capacity for the synthetic implant to grow as the patient ages which is a feature of cartilage implants (Tanzer 1978). The principle complications associated with alloplastic implants that have prevented their widespread application are that of inflammation, erosion, fracture, infection and extrusion of the implant. This process also results in significant damage to the overlying and surrounding soft tissue which may limit and even preclude the potential for subsequent autologous reconstruction. In early studies that evaluated the use of silastic implants, infection around the implants was very high and ranged from 3% - 81%, resulting in very high rates of extrusion and removal ranging from 1% - 81% (Romo et al. 2014).

The first work describing the routine use of a synthetic framework was by Cronin in 1966 (Cronin 1966). In this early work Cronin described the use of a soft silastic framework, however he acknowledged the principle problem of extrusion associated with this type of implant. Cronin initially advocated the
use of additional tissue flaps and grafts to overcome this, however when he continued to experience extrusion he subsequently abandoned the technique in favour of autologous methods.

The most common synthetic framework in current use is Medpor® which is a porous polyethylene scaffold. It was initially described in 1991 by Dr John Reinisch. In the largest published series comparing autologous and Medpor® based reconstructions, 1864 patients were studied and significantly higher extrusion rates were seen in the Medpor® group (13.5%) versus the autologous group (1%). There was also greater overall satisfaction in the autologous group versus the Medpor® group (98% versus 84% respectively) (Yanyong Zhao et al. 2009).

In another smaller study that compared the complication rates of a single surgeon using silastic versus autogenous cartilage, 81% of silastic implants required removal compared to none of the cartilage implants. Whilst this was a small study, and the total length of follow up was not recorded, these results prompted the authors to abandon the use of silastic. To minimize this risk, proponents advocate the use of a temporoparietal fascial (TPF) flap to cover the implant. However, this procedure adds to the morbidity of the procedure and often results in prominent scars and areas of hair loss within the temporal region. Using a Medpor® implant covered with a TPF flap Romo et al report a complete loss of implant in 0.8% of patients, however they
acknowledge a much higher number who encounter problems associated with the implant requiring addition treatment and surgery (Romo et al. 2014).

1.4.2.3 Prosthetics

Another alternative to autologous reconstruction is the use of prosthetic ears that can be fixed to the side of the head either with an adhesive, or by the use of bone anchored titanium screws. The advantages of this technique are that it eliminates the need for costal cartilage and the associated morbidity. It can therefore be preferable in patients who are not good candidates for lengthy surgical procedures, or who lack suitable cartilage (Thorne et al. 2001). In addition, the cosmetic appearance of prosthetic ears made by skilled and experienced prosthetists can be excellent. Despite this, there are a number of disadvantages that have limited their use. Although the ear itself may have excellent cosmetic appearance, the seam where it adjoins the head is very difficult to conceal often making the prosthesis conspicuous. In addition, the colour of the prosthetic ear is very difficult to match to the native skin and it will not respond to the changes in skin tone frequently seen throughout the year, or in response to emotions such as blushing. The need to frequently remove the prosthesis can cause wearing and failure of the implant and therefore they may need to be replaced at frequent intervals (Giot et al. 2011). The placement of titanium screws can cause local irritation and inflammation to the adjacent soft tissues. The placement of these screws and subsequent damage to the soft tissues can mean that any subsequent reconstruction is impossible (Figure 1.5).
Figure 1.5: Bilateral prosthetic ears in a patient with Treacher-Collins syndrome. A.) Anterior view. B and C.) Lateral view demonstrating poor colour match. D.) Titanium bone anchors causing tissue inflammation and erosion over the sight where subsequent reconstruction would occur. A bone anchored hearing aid is also present which can affect reconstructive options if placed incorrectly.
1.4.2.4 Acquired ear deformities

In addition to congenital deformities requiring reconstruction there are a number of acquired deformities requiring reconstruction. The most common causes of acquired deformity are trauma including bites and burns, infections, cancer and failed previous surgery such as prominent ear correction (Figure 1.6).

![Figure 1.6: Acquired ear deformities. A.) Severe deformation requiring total reconstruction and soft tissue coverage due to complications following previous surgery. B.) Partial ear loss from a human bite.](image)
1.4.2.5 The perfect solution for ear reconstruction

Reconstruction using autologous costal cartilage is regarded as the gold standard, however it is far from perfect. Even in skilled hands the reconstruction process is lengthy, expensive, exposes patients to significant morbidity and can have variable results.

Studies of costal cartilage have demonstrated wide variation in the biomechanical properties, with age related changes to cartilage such as calcification make its behavior unpredictable (Qing-Hua et al. 2011). In a study that examined the biomechanical properties of the cartilage of 90 patients, it was determined that the optimum age for reconstruction based on the biomechanical properties of the cartilage was 5-10 years. This is in keeping with that suggested by Nagata for microtia reconstruction based on chest wall circumference and the amount of cartilage required (Nagata 1993), and by Bulstrode et al based on psychological factors (Bulstrode et al. 2015). However for those patients requiring reconstruction in later life, the cartilage is likely to be suboptimal and in some instances not suitable.

It is widely acknowledged that an alternative framework that reduces the burden of rib harvest and the associated morbidity, but that behaves as autologous tissue with optimal and reproducible characteristics would be a significant advancement on current best practice. Tissue engineering may present an exciting alternative strategy for ear reconstruction that would
minimise, and potentially eliminate much of the morbidity associated with current techniques (Uppal, Sabbagh, Chana & Gault 2008a).

For auricular reconstruction, any tissue engineered solution should aim to replace the current gold standard which is costal cartilage. Native elastic cartilage of the ear lacks the required biomechanical properties to overcome forces associated with ear reconstruction and would deform under these strains. Costal cartilage has the structural integrity required maintain its 3-dimensional structure and detail whilst overcoming the forces of the overlying skin envelope. In addition, it retains sufficient elasticity and memory to overcome the daily forces applied to the ear (Qing-Hua et al. 2011). For these reasons, any solution in auricular reconstruction will require both a scaffold for structural integrity, and an appropriate cell source to generate new cartilage (Fig 1.7.1) (Y. Liu et al. 2010).

The history, principles and current state of the art in tissue engineering are discussed fully in the next section.
1.5 Tissue engineering

Tissue engineering is defined as;

“an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ”

It is estimated that over half of all money spent on healthcare in the US, is spent treating tissue or organ loss and failure (Langer & Vacanti 1993). Traditional methods used to treat these conditions include transplantation (either allograft or xenograft), surgical repair and reconstruction, artificial prostheses, mechanical devices and drugs. Whilst these treatments have saved and improved the lives of many people, they are not ideal. There is a global shortage of suitable donor organs to meet the increasing needs of patients with end stage organ failure. Surgical repair often results in suboptimal function, and has the associated morbidity and mortality of the operation. Artificial prostheses and mechanical devices may exert an inflammatory response in the host, are prone to failure and have a limited life often requiring increasingly complex procedures to replace them. Furthermore, many mechanical devices and prostheses are unable to perform all of the complex functions of the tissue or organ they aim to replace (Langer & Vacanti 1993). Tissue engineering has the potential to eliminate many of these problems as the aim is to generate identical – and where possible autologous - replacement tissue to that which is lost or damaged.
There are three general strategies when addressing the generation of new tissue (Fig 1.7). In addressing whole organ loss or failure, it is likely for a scaffold to act as a structural support whilst the cellular component replaces the lost tissue. Here the scaffold is required to have structural properties including spacial organization and mechanical strength, as well as the bioactive properties necessary to stimulate and coordinate regeneration. If the scaffold is designed to be biodegradable, it must be designed with a resorption rate that allows the mechanical strength of the scaffold to remain until the cellular component has remodeled and can resume its natural role. In addition, the potential implications of this type of construct are the need for significant periods of *in vitro* cell culture in a suitable bioreactor to attain the required number of cells prior to differentiation or implantation (Fig 1.7.1).

There are other scenarios where the aim of the scaffold is to allow the ingress of native stem cells, progenitors cells and blood vessels to repopulate the graft. Hence the role of the scaffold is to promote ingress and facilitate differentiation of the native cells eg. small tissue defects, coating of medical implants (Fig 1.7.2).

Thirdly, the aim is to deliver bioactive factors and/or cells directly to the site of injury to either stimulate, supplement, replace or modulate existing stem cell populations eg non-unions, avascular necrosis. (Fig 1.7.3).
1. “Tissue engineering” eg. Large bone/cartilage defect

2. Implant only eg. Small defects or osteoinductive / conductive layers

3. Cells / bioactive factors only eg. Non union or avascular necrosis

Figure 1.7: General strategies for tissue engineering. 1.) Cells and scaffolds for whole organ or large defect replacement. 2.) Scaffold only implants designed to stimulate and coordinate native repair and regeneration. 3.) Delivery of cells and/or bioactive factors to stimulate regeneration.

Despite the interest in tissue engineering strategies to address significant health problems, the application in clinical practice is not routine and is limited to small case reports and series. The majority of these clinical applications involved relatively ‘simple’ tissue types such bone and cartilage that do not perform complex functions such as major organs. However, more recently the ability to grow complex organized and functional organs from stem cells has been described in vivo (Bredenkamp et al. 2014). In the case of auricular engineering, there are significant forces applied upon the
reconstructed ear when it is placed under the skin envelope, therefore the biomechanical properties of any scaffold must be sufficient to overcome these forces until adequate replacement tissue has been generated. Tissue engineering for auricular reconstruction is discussed fully in Chapter 1.8.

1.6 Biomaterials

Biomaterials are any materials that interface with biological systems, and can include ceramics, metals, glass, polymers and hydrogels. The use of synthetic materials in medicine is well established and has significantly improved the quality of life in patients suffering from tissue loss and degeneration. To date the majority of these implants used to replace damaged tissue are orthopaedic implants made from metals and ceramics.

Initial biomaterial and implant research focused on finding inert substrates that could replace lost or damaged tissue, but evoke minimal effects on the native tissue. Coupled with advances in cell based therapies and the emergence of regenerative medicine, there has been a significant shift in focus from looking at the application of inert materials that replace lost and damaged tissue, to trying to identify biocompatible and bioactive materials that can stimulate and co-ordinate the replacement and regeneration of tissue (McMurray et al. 2011).

Whilst metals and ceramics are widely used in orthopaedics to replace diseased joints from degenerative diseases such as arthritis, their application
as a biomaterial to regenerate cartilage and support chondrogenesis has been largely unsuccessful due to undesirable biomechanical properties (Seal et al. 2001).

The ability to accurately and reproducibly engineer a material to its targeted purpose and end use makes synthetic polymers and hydrogels ideal candidates as biomaterials for cartilage tissue repair and regeneration. Critically for chondrogenesis and cartilage tissue engineering, the stiffness, elasticity, load bearing capacity, biodegradability, surface structure and chemistry, and can all be tailored to the desired outcome. Polymers and hydrogels have therefore been the principle biomaterials investigated for their use in chondrogenesis and cartilage tissue engineering (Puppi et al. 2010)

Hydrogels are three-dimensional structures formed by the crosslinking of either natural or synthetic homopolymers, copolymers or macromers. Their hydrophilic structure allows them to retain amounts of water within their 3-D networks. Hydrogels have been extensively employed in tissue engineering and regenerative strategies due to their ability to imitate the native extracellular matrix and the stem cell niche (S. Q. Liu et al. 2010). The high water content of hydrogels results in excellent biocompatibility and biodegradability and the mechanical properties can be easily manipulated.
(Alakpa et al. 2016). This makes hydrogels an ideal candidate for tissue regeneration and cellular support.

Natural hydrogels include proteins found in the ECM of human tissues such as collagen and hyalauronic acid (HA), proteins derived from plants and algae such as cellulose and alginate, and those derived from animal cell lines such as Matrigel which contains ECM proteins produced from a mouse sarcoma cell line.

Polymers are the most widely used biomaterials and have been utilized in a diverse range of applications including sutures, heart valves, surgical mesh and screws. They are excellent candidates for biomaterials as they are easy to manufacture and produce in a cost efficient manner with highly reproducible characteristics. Polymers are incredibly versatile and can be manipulated to have the desired physical and mechanical properties for their intended application. They can be manufactured in many different forms including solids, gels, liquids and fibres. In addition, they can be used directly, in combination with other materials or coated onto surfaces (Seal et al. 2001). They can be both biodegradable and bioactive, with many examples of polymers supporting and enhancing differentiation of stem and progenitor cells into hepatic (Hay et al. 2011), bone (F. Khan et al. 2010) and endothelial lineages (Pernagallo et al. 2012).

Polymers have also been tested for their capacity to support chondrogenesis of stem cell populations and specifically auricular tissue engineering (Shieh
et al. 2004). The commonest group of synthetic polymers used for auricular tissue engineering are aliphatic polyesters such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and poly(caprolactone) (PCL), which are biodegradable through hydrolysis of the ester linkages. These groups have been shown to be biocompatible and support the growth and differentiation of both chondrocytes and stem cell populations (Cao et al. 1997). Problems arising with these polymers come from acidic degradation products that can cause resorption of the engineering structure, immunogenicity of the degradation products (Nayyer et al. 2012), and insufficient biomechanical strength to maintain the shape of the engineered structure long term in vivo (Shieh et al. 2004) (Cao et al. 1997). Blending of different polymers allows for alterations in their degradation rates and has demonstrated improved biomechanical function in longer term studies of 20 weeks in vivo implantation (Pomerantseva et al. 2016).

Table 1.2 summarises some of the most common classes of polymers in use in musculoskeletal therapies (Puppi et al. 2010; Seal et al. 2001).
<table>
<thead>
<tr>
<th>Polymer</th>
<th>Musculoskeletal applications</th>
<th>Features / Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyphosphazene</td>
<td>Bone cements, tissue engineering.</td>
<td>Biodegradable and highly tunable with changes in side groups. Comparable effect to PLGA when tested \textit{in vivo}. Able to neutralize acidic degradation products of other materials.</td>
</tr>
<tr>
<td>Poly (α-hydroxyacids) (PLA, PLLA, PLGA, PGA)</td>
<td>Tissue engineering, drug delivery (also used as sutures, stents, dressings).</td>
<td>Most widely used and investigated biomaterials. Highly tunable and easy to combine with other materials. Acidic degradation products cause strong inflammatory response. Random degradation can lead to premature failure of scaffolds. Hydrophobic and therefore needs modification to support cellular adhesion. PLA scaffolds have been used in auricular tissue engineering but unable to support 3-D structure \textit{in vivo} over long term.</td>
</tr>
<tr>
<td>Poly (L-lactide-co-e-caprolactone) (PCL)</td>
<td>Multiple uses including intramedullary fracture pins, craniofacial repair, bone and cartilage regeneration, tissue engineering.</td>
<td>Biodegradable polymer with FDA approval as medical device. Many uses on own or often in combination with other materials such as ceramics (added strength and mineralization). Has been used in combination with PLA to engineer auricular strutures that demonstrate sufficient mechanical integrity in long term \textit{in vivo} implantation.</td>
</tr>
<tr>
<td>Poly (propylene fumarate) PPF</td>
<td>Tissue engineering</td>
<td>Biodegradable, however acidic degradation products can cause inflammatory reaction. Highly tunable with variable crosslinking. Liquid before crosslinking therefore easy to fabricate into custom made shapes or directly inject. Good biomechanical</td>
</tr>
</tbody>
</table>

Poly (1,4-butylene succinate) PBSu & Tissue engineering & Biodegradable with harmless degradation products. Able to support attachment, proliferation and phenotype of osteoblasts when manufactured appropriately. 

Poly (Acrylate)s & Cartilage and bone replacement, tissue engineering & Nondegradable. Many individual polyacrylates can be prepared from a huge range of monomers. Excellent tunability. Favourable biomechanical properties. Able to support stem and progenitor cell attachment, proliferation and differentiation.

| Table 1.2. A summary of the most common polymers in clinical use. Adapted from Puppi et al, 2010, and Seal et al, 2001. |

1.7 Cells for tissue engineering

Early research into tissue engineering frequently used mature cells such as chondrocytes or epithelial cells (Cao et al. 1997). Mature tissue cells are capable of generating new tissue, however there are limitations to their use. Donor sites are often limited and also may be affected by the disease they are trying to treat such as arthritis in chondrocytes. Furthermore, cells require extended periods of in vitro culture prior to use and mature cells have been shown to have limited ability to expand in vitro with associated deterioration in the function and the potential for genetic instability (Yimu Zhao et al. 2012).
Stem cells are defined as cells with the capacity to self-renew and in response to intrinsic and extrinsic factors, differentiate into multiple cell types with specialised function. The potency of a stem cell refers to the number of different cells/tissues a particular cell is able to differentiate into. For example, a totipotent cell from a newly fertilised morula is capable of differentiating into all intra and extra embryonic tissues, where as a multipotent cell is capable of differentiating into cells form within its own germ layer such as mesenchymal stem cells (Figure 1.8).

Stem cells have generated great interest for the use in tissue engineering. Whilst mature tissue cells have limited ability to expand in culture, stem cells by their nature are capable of prolonged periods of expansion and culture with no significant change in their function. In addition, stem cells can be harvested from multiple different sources (Covas et al. 2008). Although embryonic stem cells are able to generate all tissue types, they have a number of ethical and practical implications that have limited their use in tissue engineering. Conversely, adult stem cells have a narrower potency, but are potentially better suited to clinical translation. They can be harvested relatively easily from autologous sources and have not demonstrated any capacity for teratoma formation or malignant transformation (Bernardo et al. 2007).
Figure 1.8: Hierarchy of potency of stem cells. Totipotent cells of the morula capable of generating all intra and extra embryonic tissues. Pluripotent cells from the inner mass of the blastocyst capable of generating all cell types within the embryo. Multipotent cells capable of generating cells from within a specific germ layer. Unipotent cells capable of generating a single terminally differentiated cell type.
1.7.1 Mesenchymal stem cells

The presence of non-haematopoietic stem cells in the bone marrow was first described by Julius Conheim, a German pathologist in 1867 (Prockop 1997). Using an analine dye, he observed the presence of non-haematopoietic fibroblast like cells in wounds created at distal sites. He concluded that these cells had migrated from the bone marrow, and that bone marrow was thus a source of non-inflammatory cells that contributed to wound healing.

It was the pioneering work of the Russian scientist Friedenstein in the 1960s who first demonstrated that these bone marrow derived, adherent, non-haematopoietic, fibroblast like cells had the ability to form colonies and differentiate into bone and cartilage in vitro, and could generate bone when transplanted to ectopic locations in vivo (Friedenstein et al. 1966; Friedenstein et al. 1970; Friedenstein et al. 1974). Furthermore, in models of serial implantation they were able to regenerate heterotopic bone in vivo demonstrating their capacity for self renewal (Owen & Friedenstein 1988). Due to their appearance in vitro, Friedenstein referred to these cells as colony forming unit – fibroblast (CFU-F), a name that was routinely used before Caplan introduced the term Mesenchymal Stem Cell (MSC) in 1991 (Caplan 1991).

Since their initial description, these cells have been the focus of much attention for their ability to differentiate into multiple mesodermal lineages, to modulate the immune system and to stimulate regeneration through trophic
support and the secretion of cytokines (Caplan 2007). MSC have the general perception of being ‘safe’ stem cells due to the fact that these cells can be harvested relatively easily from autologous donors and have not demonstrated any evidence of tumour formation in vitro or in vivo, one of the principle concerns with the use of pluripotent cells. In addition, MSC can be selected and rapidly expanded using standard culture methods and are reported to have a broad therapeutic action. It is not therefore surprising that MSC have been the focus of a number of clinical trials, many of which have aimed to demonstrate a function beyond just mesenchymal repair and regeneration (www.clinicaltrials.gov). Despite many trials, the application of MSC in routine clinical practice is not currently prevalent. This is possibly due to disappointing trial outcomes, and the high cost of manufacturing cells to the standards required for clinical application. Despite this, there is still much promise for the application of these cells to treat a range of diseases, and they are the most frequently used cell in research focusing on chondrogenesis and cartilage repair (Table 1.3).
<table>
<thead>
<tr>
<th>Disease</th>
<th>I</th>
<th>I/II</th>
<th>II</th>
<th>II/III</th>
<th>III</th>
<th>IV</th>
<th>ND</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>31</td>
<td>40</td>
<td>15</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Haematological disease</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GVHD</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Diabetes</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Liver disease</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Kidney disease</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Lung disease</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>2</td>
<td>11</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Bone and cartilage disease</td>
<td>12</td>
<td>8</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Neurological disease</td>
<td>9</td>
<td>8</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Lupus erythematosus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 1.3 Completed clinical trials of MSC. Table giving details of completed MSC based clinical trials with details of the disease type and phase of trial. The majority of trials (81/104) are focused on tissues of non-mesodermal origin suggesting a trophic mode of action rather than direct contribution of MSC. Data from www.clinicaltrials.gov

1.7.2 Defining MSC

Due to the widespread interest in MSC, there have been many attempts made to standardise and simplify the nomenclature associated with these cells. However, there has been significant variation used to isolate and grow MSC, and also in the assays used to examine their function. This has made interpreting and standardising information on MSC both challenging and potentially misleading (Tallone et al. 2011). In an attempt to address these issues, the Mesenchymal and Tissue Stem Cell Committee of The International Society for Cellular Therapy (ISCT) published a position
statement that addressed the minimum criteria required to define an MSC (Dominici et al. 2006). They stated that cells must:

- Be adherent to plastic in standard conditions.
- Have positive (>95%) expression of CD105, CD90, CD73.
- Have negative (<2%) expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR.
- \textit{In vitro} differentiation into osteoblasts, adipocytes and chondroblasts by demonstration of staining of cell cultures.

Whilst the position statement published by ISCT aimed to clarify some of the issues surrounding MSC identity, subsequent studies have demonstrated variable expression of the markers described by the ISCT and that many of these markers appear to be neither inclusive nor exclusive of MSC function. In addition, many subsequent studies have identified many new and different cell surface antigens that are associated with MSC (summarized in table 1.4, reviewed in (da Silva Meirelles 2006; Murray et al. 2013; Crisan et al. 2008)). It is therefore recognized that MSC selected according to their ability to adhere to plastic represent a heterogenous pool of cells with significant variation in their immunophenotype and function. Attention has focused on identifying surface markers that might represent functionally distinct subsets with enhanced potential. STRO-1 has been identified as a marker that represents a subset of bone marrow derived MSC with enhanced clonogenicity which contains osteoprogenitors (Arai 2002; Gronthos et al.
1994; Simmons & Torok-Storb 1991). If cells expressing STRO-1 and VCAM-1 were selected, this effect was enhanced (Gronthos 2003). A subset of MSC from human intrapatellar fat pads that expressed the pericyte marker 3G5 demonstrated enhanced chondrogenesis in vitro, interestingly these cells were negative for STRO-1 expression further supporting the hypothesis that MSC contain subsets of cells with enhanced ability toward a specific function.

One problem associated with assessing surface marker expression in vitro is that many of these markers can be modulated by culture conditions, and they play no direct role in the underlying fate decision relating to self renewal and differentiation. For example, CD105 is one of the surface markers that is defined in the ISCT minimal criteria, however studies have demonstrated that expression of this marker is inversely related to the osteogenic potential of MSC (Levi et al. 2011). In addition, Jones et al demonstrated lines of MSC with uniform expression of HLA-DR (a marker that should not be expressed according to the ISCT criteria) which co-expressed CD90 and CD105 and were capable of multi-lineage differentiation (Jones et al. 2002). Therefore although numerous markers have been reported as being either expressed or negatively expressed in MSC, there is very little data about how this relates to function, or in vivo identity of cells.
<table>
<thead>
<tr>
<th>CD Marker</th>
<th>+/-</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td></td>
<td>(Mariotti et al. 2008)</td>
</tr>
<tr>
<td>CD9</td>
<td></td>
<td>(Gronthos et al. 2001; Niehage et al. 2011; Mariotti et al. 2008; Gimble et al. 2007)</td>
</tr>
<tr>
<td>CD10</td>
<td>+</td>
<td>(Gronthos et al. 2001; Crisan et al. 2008)</td>
</tr>
<tr>
<td>CD11a</td>
<td>-</td>
<td>(Pittenger et al. 1999; Dominici et al. 2006)</td>
</tr>
<tr>
<td>CD11b</td>
<td>-</td>
<td>(Dominici et al. 2006)</td>
</tr>
<tr>
<td>CD13</td>
<td>+</td>
<td>(Gronthos et al. 2001; Crisan et al. 2008)</td>
</tr>
<tr>
<td>CD14</td>
<td>-</td>
<td>(Kern et al. 2006; Dominici et al. 2006; Pittenger et al. 1999; Niehage et al. 2011)</td>
</tr>
<tr>
<td>CD16</td>
<td>-</td>
<td>(Mariotti et al. 2008)</td>
</tr>
<tr>
<td>CD18</td>
<td>+</td>
<td>(Brooke et al. 2008)</td>
</tr>
<tr>
<td>CD19</td>
<td>-</td>
<td>(Pittenger et al. 1999; Dominici et al. 2006)</td>
</tr>
<tr>
<td>CD27</td>
<td>-</td>
<td>(Mariotti et al. 2008)</td>
</tr>
<tr>
<td>CD28</td>
<td>-</td>
<td>(Mariotti et al. 2008)</td>
</tr>
<tr>
<td>CD31</td>
<td>-</td>
<td>(Brooke et al. 2008; Mariotti et al. 2008; Gimble et al. 2007; Tallone et al. 2011)</td>
</tr>
<tr>
<td>CD33</td>
<td>-</td>
<td>(Mariotti et al. 2008)</td>
</tr>
<tr>
<td>CD36</td>
<td>-</td>
<td>(Mariotti et al. 2008)</td>
</tr>
<tr>
<td>CD44</td>
<td>+</td>
<td>(Gronthos et al. 2001; Niehage et al. 2011; Brooke et al. 2008; Mariotti et al. 2008; Gimble et al. 2007; Wagner et al. 2005; Kern et al. 2006; Crisan et al. 2008)</td>
</tr>
<tr>
<td>CD45</td>
<td>-</td>
<td>(Kern et al. 2006; Dominici et al. 2006; Niehage et al. 2011; Mariotti et al. 2008; Gimble et al. 2007)</td>
</tr>
<tr>
<td>CD49A</td>
<td>+</td>
<td>(Brooke et al. 2008; Mariotti et al. 2008; Gimble et al. 2007; Gronthos et al. 2001)</td>
</tr>
<tr>
<td>CD49B</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>CD49C</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>CD49E</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>CD50</td>
<td>-</td>
<td>(Brooke et al. 2008)</td>
</tr>
<tr>
<td>CD51</td>
<td>+</td>
<td>(Brooke et al. 2008)</td>
</tr>
<tr>
<td>CD54</td>
<td>+</td>
<td>(Gronthos et al. 2001; Niehage et al. 2011; Brooke et al. 2008; Gimble et al. 2007)</td>
</tr>
<tr>
<td>CD55</td>
<td>+</td>
<td>(Gimble et al. 2007; Gronthos et al. 2001)</td>
</tr>
<tr>
<td>CD56</td>
<td>+</td>
<td>(Brooke et al. 2008; Tallone et al. 2011)</td>
</tr>
<tr>
<td>CD58</td>
<td>+</td>
<td>(Mariotti et al. 2008)</td>
</tr>
<tr>
<td>CD59</td>
<td>+</td>
<td>(Gronthos et al. 2001; Campioni et al. 2006)</td>
</tr>
<tr>
<td>CD61</td>
<td>+</td>
<td>(Niehage et al. 2011)</td>
</tr>
<tr>
<td>CD63</td>
<td>+</td>
<td>(Niehage et al. 2011)</td>
</tr>
<tr>
<td>CD71</td>
<td>+</td>
<td>(Niehage et al. 2011; Mariotti et al. 2008; Gimble et al. 2007)</td>
</tr>
<tr>
<td>CD73</td>
<td>+</td>
<td>(Dominici et al. 2006; Mariotti et al. 2008; Gimble et al. 2007; Wagner et al. 2005; Kern et al. 2006; Crisan et al. 2008)</td>
</tr>
<tr>
<td>CD79a</td>
<td>-</td>
<td>(Dominici et al. 2006)</td>
</tr>
<tr>
<td>CD90</td>
<td>+</td>
<td>(Dominici et al. 2006; Niehage et al. 2011; Mariotti et al. 2008; Gimble et al. 2007; Wagner et al. 2005; Kern et al. 2006; Tallone et al. 2011; Crisan et al. 2008)</td>
</tr>
<tr>
<td>-------</td>
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<td>-----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>CD97</td>
<td>+</td>
<td>(Niehage et al. 2011)</td>
</tr>
<tr>
<td>CD98</td>
<td>+</td>
<td>(Niehage et al. 2011)</td>
</tr>
<tr>
<td>CD99</td>
<td>+</td>
<td>(Niehage et al. 2011)</td>
</tr>
<tr>
<td>CD102</td>
<td>-</td>
<td>(Brooke et al. 2008)</td>
</tr>
<tr>
<td>CD104</td>
<td>+</td>
<td>(Brooke et al. 2008)</td>
</tr>
<tr>
<td>CD105</td>
<td>+</td>
<td>(Dominici et al. 2006; Gronthos et al. 2001; Niehage et al. 2011; Gimble et al. 2007; Kern et al. 2006; Crisan et al. 2008)</td>
</tr>
<tr>
<td>CD106</td>
<td>+</td>
<td>(Gronthos et al. 2001; Niehage et al. 2011; Brooke et al. 2008; Kern et al. 2006)</td>
</tr>
<tr>
<td>CD117</td>
<td>-</td>
<td>(Zimmerlin et al. 2009)</td>
</tr>
<tr>
<td>CD120A</td>
<td>+</td>
<td>(Pittenger et al. 1999)</td>
</tr>
<tr>
<td>CD124</td>
<td>+</td>
<td>(Pittenger et al. 1999)</td>
</tr>
<tr>
<td>CD133</td>
<td>-</td>
<td>(Kern et al. 2006; Niehage et al. 2011)</td>
</tr>
<tr>
<td>CD140</td>
<td>+</td>
<td>(Bühring et al. 2007; Masuda et al. 2012; Crisan et al. 2008; Tallone et al. 2011)</td>
</tr>
<tr>
<td>CD144</td>
<td>-</td>
<td>(Kern et al. 2006; Crisan et al. 2008)</td>
</tr>
<tr>
<td>CD166</td>
<td>+</td>
<td>(Gronthos et al. 2001; Niehage et al. 2011; Brooke et al. 2008; Gimble et al. 2007; Wagner et al. 2005)</td>
</tr>
<tr>
<td>CD243</td>
<td>-</td>
<td>(Mariotti et al. 2008)</td>
</tr>
<tr>
<td>CD271</td>
<td>+</td>
<td>(Tallone et al. 2011; Bühring et al. 2007; Flores-Torales et al. 2010)</td>
</tr>
<tr>
<td>CD276</td>
<td>+</td>
<td>(Niehage et al. 2011)</td>
</tr>
<tr>
<td>CD304</td>
<td>+</td>
<td>(Niehage et al. 2011)</td>
</tr>
<tr>
<td>CD324</td>
<td>+</td>
<td>(Brooke et al. 2008)</td>
</tr>
<tr>
<td>CD340</td>
<td>+</td>
<td>(Bühring et al. 2007)</td>
</tr>
<tr>
<td>CD349</td>
<td>+</td>
<td>(Bühring et al. 2007)</td>
</tr>
<tr>
<td>αSMA</td>
<td>+</td>
<td>(Crisan et al. 2008; Tallone et al. 2011)</td>
</tr>
<tr>
<td>NG2</td>
<td>+</td>
<td>(Tallone et al. 2011; Crisan et al. 2008)</td>
</tr>
<tr>
<td>STRO-1</td>
<td>+</td>
<td>(Dennis et al. 2002; Simmons &amp; Torok-Storb 1991; Gronthos et al. 1994)</td>
</tr>
</tbody>
</table>

**Table 1.4. Surface markers associated with MSC.** A list of published studies demonstrating positive (+), and negative (-) expression of surface markers. Almost all these studies relate to in vitro studies and provide little evidence about the relative function of MSC or their in vivo identity.
1.7.3 Functions of MSC

In addition to their ability to differentiate into multiple mesodermal tissues, MSC also have been shown to have a number of further functions that make them attractive therapeutic targets.

In his initial series of experiments, Friedenstein demonstrated the vital role that MSC play in the support of haematopoiesis. In these experiments, MSC (called CFU-F in the work), were transplanted into heterotopic locations in semi-syngeneic animals. The formation of heterotopic ossicles was observed with the haematopoietic cells being of recipient origin, but the bone forming cells being donor derived. Friedenstein concluded that MSC provided an environment for HSC homing, engraftment and subsequent establishment of haematopoiesis (Friedenstein et al. 1974).

The long term ability of MSC to support haematopoiesis was demonstrated in an in vitro murine model by Dexter et al (Dexter et al. 1977). Culture systems were established demonstrating the maintainance of haematopoiesis over several months. Within these cultures of MSC it was shown that subsets expressing the STRO-1 antigen were those capable of supporting haematopoiesis, whilst also having the ability to differentiate into multiple mesenchymal lineages (Simmons & Torok-Storb 1991; Dennis et al. 2002).

Based on this early experimental work, MSC have been investigated extensively for their ability to support haematopoiesis and promote HSC
engraftment and repopulation in vitro and in vivo. Co-transplantation of HSC with MSC has been shown to increase the haematopoietic engraftment and recovery in both animal and human studies (Bensidhoum et al. 2004; Devine et al. 2001).

Immunomodulation is another key property displayed by MSC, and this immunosuppression has allowed allogeneic transplantation of these cells, avoiding host immune surveillance and without obvious immune response from the host (Aggarwal 2005; Wada et al. 2013). The immunomodulatory properties of MSC are mediated via the secretion of bioactive molecules affecting dendritic cells, B Cells and T Regulatory, Helper and Killer cells, and also by direct cell-to-cell contact (Aggarwal 2005; Caplan & Correa 2011). The exact mechanisms by which MSC exert this effect remains to be elucidated, however several studies have demonstrated numerous different factors that may play a role. MSC have been shown to release immunomodulatory biomolecules such as IL-10, interferon-γ, indoleamine 2,3-dioxygenase which prevented arteriosclerosis and enhanced graft survival in a porcine model of femoral artery transplantation (Jui et al. 2012). In a porcine model of hind limb composite tissue allotransplantation, administration of autologous MSC resulted in significantly increased graft survival associated with T-cell regulation (Kuo et al. 2012). Furthermore the immunosuppressive effects of MSC where shown to have a chondroprotective function in a murine model of arthritis. Following injection
of MSC into affected joints, T cell numbers were reduced and disease progression slowed (Wu et al. 2016).

Many of the studies examining the therapeutic function of MSC have observed benefits that extend beyond differentiation into mesodermal lineages alone. This broad therapeutic action of MSC may be as a result of the vast secretome they possess and has lead to MSC being referred to as ‘an injury drugstore’ (Caplan & Correa 2011). This therapeutic function has been examined in over 100 completed and nearly 500 on going clinical trials, and in the vast majority of these trials the proposed mechanism of action has been attributed to immunomodulation and secretion of trophic factors and not direct contribution to new tissues (Table 1.3 & 1.5)

<table>
<thead>
<tr>
<th>Disease</th>
<th>Phase of trial</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Haematological disease</td>
<td>4</td>
</tr>
<tr>
<td>GVHD</td>
<td>4</td>
</tr>
<tr>
<td>Diabetes</td>
<td>7</td>
</tr>
<tr>
<td>Liver disease</td>
<td>4</td>
</tr>
<tr>
<td>Kidney disease</td>
<td>0</td>
</tr>
<tr>
<td>Lung disease</td>
<td>9</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>16</td>
</tr>
<tr>
<td>Bone and cartilage disease</td>
<td>19</td>
</tr>
<tr>
<td>Neurological disease</td>
<td>21</td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td>2</td>
</tr>
<tr>
<td>Lupus erythematos</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>20</td>
</tr>
</tbody>
</table>

**Table 1.5 All currently open clinical trials of MSC.** Table giving details of all 493 currently open MSC based clinical trials with details of the disease type and phase of trial. Data from www.clinicaltrials.gov.
1.7.4 MSC in multiple tissues

Since their initial isolation from bone marrow, seemingly identical cells have been isolated from almost all foetal and post natal tissues based on their adherence to plastic (da Silva Meirelles 2006) (Table 1.5). This includes organs that contain no native mesenchymal tissue, further supporting the hypothesis that these cells have a function beyond just mesodermal differentiation.

<table>
<thead>
<tr>
<th>Human Tissue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>(da Silva Meirelles 2006)</td>
</tr>
<tr>
<td>Adipose</td>
<td>(Zuk et al. 2002; Crisan et al. 2008; De Ugarte et al. 2003)</td>
</tr>
<tr>
<td>Amniotic Fluid</td>
<td>(Tsai et al. 2004; In’t Anker et al. 2004)</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>(Friedenstein et al. 1966; Friedenstein et al. 1974; Friedenstein et al. 1970; Pittenger et al. 1999; Shi &amp; Gronthos 2003; da Silva Meirelles 2006; Crisan et al. 2008; Campagnoli 2001)</td>
</tr>
<tr>
<td>Blood</td>
<td>(Villaron et al. 2004)</td>
</tr>
<tr>
<td>Brain</td>
<td>(da Silva Meirelles 2006; Crisan et al. 2008; Paul et al. 2012)</td>
</tr>
<tr>
<td>Cartilage</td>
<td>(Alsalameh et al. 2004; Hiraoka et al. 2006)</td>
</tr>
<tr>
<td>Endometrium</td>
<td>(Schuring et al. 2011; Spitzer et al. 2012)</td>
</tr>
<tr>
<td>Eyes</td>
<td>(Crisan et al. 2008)</td>
</tr>
<tr>
<td>Gut</td>
<td>(Lanzoni et al. 2009; Crisan et al. 2008)</td>
</tr>
<tr>
<td>Heart</td>
<td>(Crisan et al. 2008)</td>
</tr>
<tr>
<td>Kidney</td>
<td>(da Silva Meirelles 2006)</td>
</tr>
<tr>
<td>Liver</td>
<td>(Campagnoli 2001; da Silva Meirelles 2006)</td>
</tr>
<tr>
<td>Lungs</td>
<td>(da Silva Meirelles 2006; Crisan et al. 2008)</td>
</tr>
<tr>
<td>Muscle</td>
<td>(Asakura et al. 2001; da Silva Meirelles 2006; Crisan et al. 2008)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>(da Silva Meirelles 2006; Crisan et al. 2008)</td>
</tr>
<tr>
<td>Perichondrium</td>
<td>(Arai 2002)</td>
</tr>
<tr>
<td>Periodontal</td>
<td>(da Silva Meirelles 2006; Seo et al. 2004)</td>
</tr>
<tr>
<td>Tissue</td>
<td>References</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Ligament</td>
<td>(Zuk et al. 2002; Igura et al. 2004; Crisan et al. 2008; In’t Anker et al. 2004; De Ugarte et al. 2003)</td>
</tr>
<tr>
<td>Placenta</td>
<td>(Friedenstein et al. 1966; Tsai et al. 2004; Friedenstein et al. 1974; Rotter et al. 2008; Friedenstein et al. 1970; In’t Anker et al. 2004; Pittenger et al. 1999; Shi &amp; Gronthos 2003; da Silva Meirelles 2006; Crisan et al. 2008; Campagnoli 2001)</td>
</tr>
<tr>
<td>Skin</td>
<td>(Villaron et al. 2004; Friedenstein et al. 1966; Toma et al. 2001; Friedenstein et al. 1974; Crisan et al. 2008; Friedenstein et al. 1970; Pittenger et al. 1999; Shi &amp; Gronthos 2003; da Silva Meirelles 2006; Campagnoli 2001)</td>
</tr>
<tr>
<td>Spleen</td>
<td>(da Silva Meirelles 2006; Villaron et al. 2004; Crisan et al. 2008; Paul et al. 2012)</td>
</tr>
<tr>
<td>Tendon</td>
<td>{Salingcarnboriboon:2003hs}</td>
</tr>
</tbody>
</table>

Table 1.5. Human tissues from which MSC have been isolated.

Whilst MSC derived from different tissues appear broadly similar and fulfill the criteria specified by the ISCT, differences in their morphology, immunophenotype and function have been observed. Significant variation in the surface marker profile has been observed between cells from different sources (Brooke et al. 2008; Gimble et al. 2007; Niehage et al. 2011), however different methods of isolation, culture and assessment make direct
comparison of results difficult (Nery et al. 2012). Clonal analysis of cell populations varies between donors and tissues and the frequency of tripotent cells can range from 30 – 50% (Guilak et al. 2005).

It has been demonstrated in a murine model that the progenitors of white adipose tissue reside in the blood vessel wall. Although these cells are found in blood vessels throughout the body, it is only those found in adipose tissue that can differentiate into adipocytes suggesting a tissue specific role of MSC (Tang et al. 2008). When examining MSC from bone marrow and adipose tissue it was seen that MSC preferentially differentiated into osteocytes and adipocytes respectively, again suggesting a tissue specificity (Peng et al. 2008). With reference to chondrogenesis, it has been demonstrated that in standard conditions MSC from bone marrow have a greater chondrogenic potential than those from adipose tissue (Diekman et al. 2010). However, these were not matched samples, and subsequent studies have shown that this difference can be eliminated with modifications to the culture medium used with the supplementation of additional growth factors (Hennig et al. 2007).

1.7.5 Stem cells in adipose tissue

In 2001, Zuk et al aimed to establish if adipose tissue contained populations of stem cells that could be used for clinical application (Zuk et al. 2001). They noted the ethical issues surrounding ES cells, and the practical implications of harvesting and expanding bone marrow derived MSC. As uncommitted
MSC had been observed in the connective tissue of multiple organs from different mammalian species, and bone marrow and adipose tissue both derive from mesoderm and contain heterogeneous stromal cell populations, they hypothesized that adipose tissue may harbor populations of MSC.

Adipose tissue was enzymatically dissociated and centrifuged to generate SVF, before being cultured under standard conditions. Cells rapidly attached and expanded in vitro and had typical fibroblast like appearance – in this experiment they were termed Processed Lipoaspirate cells (PLA). These cells continued to stably proliferated in vitro for extended periods (>165days) showing minimal signs of senescence. When placed in appropriate media and conditions they demonstrated multilineage potential and differentiated into osteogenic, chondrogenic, adipogenic and myogenic lineages in a manner identical to the bone marrow MSC controls. They concluded that adipose tissue is an autologous source of multipotent MSC, that can be easily harvested in significant numbers making them excellent candidates for tissue engineering and cell based therapies (Zuk et al. 2001). The principle advantage of adipose tissue when compared to other sources of MSC is the ability to harvest large volumes of otherwise redundant tissue with minimal morbidity, even in patients of healthy BMI. The function of ASC has been investigated in numerous studies and they have been shown to have a function broadly identical to MSC from other sources (Sterodimas et al. 2010).
1.7.6 MSC in vivo

The study of MSC has almost exclusively been observed *in vitro* with little understanding of their native location and identity. Therefore despite our perceived expansion in understanding these cells, their native role in development, homeostasis, repair and regeneration has been largely overlooked.

In early development there is a huge recruitment, expansion, migration and differentiation of stem cells; however this process is relatively short lived and decreases throughout the life of an organism. In adult organisms the frequency of stem cells is rare and the ability of tissues to repair and regenerate becomes increasingly diminished. Adult stem cells are known to live in specific niches in their corresponding tissue of origin such as HSC in the bone marrow, epidermal stem cells in hair follicles and neural stem cells in the subventricular zone (Watt & Hogan 2000). Despite isolating MSC from multiple different and distinct tissue types, identifying their native location and niche proved challenging. The main tissue types that are believed to derive from MSC are not limited to one anatomical location (e.g., bone, muscle, fat and cartilage), therefore for MSC to be able to repair and regenerate these tissues, they must either be locally available or able to mobilise and reach distant sites.

For MSC to be able to mobilise and reach distant sites, it is intuitive that these cells would be present in peripheral blood, however identifying and
establishing growth of MSC cultures from blood has proved extremely difficult even after stimulation with cytokines (da Silva Meirelles 2006; Lazarus et al. 1997). In a rat model it was observed that low oxygen conditions resulted in the specific mobilisation of MSC detected in peripheral blood (Rochefort et al. 2006), and raised numbers of MSC were also observed in the blood of patients following traumatic hip injury (Alm et al. 2010), however in both cases this elevation was modest.

Therefore the alternative hypothesis that MSC are locally available and ubiquitous throughout the body was proposed. The ability to derive functionally and phenotypically similar cells from multiple tissues supports the hypothesis that these cells may share the same in vivo location or niche throughout the body.

1.7.7 A perivascular origin of MSC

Based on the hypothesis that MSC share a ubiquitous niche throughout the body, groups have demonstrated MSC like characteristics of distinct subsets of cells isolated from blood vessel walls. Pericytes (also known as mural or Rouget cells) reside on the abluminal surface of endothelial cells and are recognised as a distinct cellular entity with a unique immunophenotype (Sims 1986). In a comprehensive examination of pericytes in multiple foetal and adult tissues, Crisan et al demonstrated that pericytes express specific perivascular surface markers (CD146, NG2, PDGFRβ), they also natively express MSC associated markers (CD29, CD44, CD73, CD90, CD105, alkaline phosphatase), and lack expression of markers specific of endothelial
(CD31, CD34, CD144, von Willebrand Factor) or haematopoietic lineages (CD34, CD45) (Crisan et al. 2008) (Figure 1.9). These results have been confirmed in further studies and are summarized in Table 1.6.

Figure 1.9: Identification of pericytes in multiple human tissues. Immunohistochemical analysis of multiple human tissues. A = myocardium, B, D, G - I = skeletal muscle, C & F = placental villus, E = pancreas. Figure taken from (Crisan et al. 2008)

In addition, it has been demonstrated that pericytes have a differentiation potential identical to that of conventional MSC (Covas et al. 2008; Crisan et al. 2008), and are able to differentiate into bone (James, Zara, Corselli, Askarinam, et al. 2012; James, Zara, Corselli, Chiang, et al. 2012), fat (Zimmerlin et al. 2012), cartilage (Crisan et al. 2008; Farrington-Rock 2004; W. S. Khan et al. 2008) and skeletal muscle (Crisan et al. 2009; Park et al.
2011). Other functions commonly attributed to MSC include the ability to suppress T-lymphocyte function which has also been observed in studies examining pericytes (Tu et al. 2011; Maier & Pober 2011), and support of haematopoiesis (Corselli, Chin, et al. 2013).

By performing a rigorous analysis of different subsets of cells within SVF of multiple tissues Corselli et al identified a second population of perivascular cells with MSC like features capable of multi lineage differentiation. These cells reside in the adventitial layer of blood vessels (adventitial cells) and have a unique surface marker profile distinct from pericytes (CD34+, CD146-, CD31-, CD45-) (Corselli et al. 2012). No other cells were found outside of these 2 distinct populations within the SVF that displayed any MSC features.

In studies by other groups that have examined subsets of stem cells within SVF similar populations were confirmed. Zimmerlin et al performed an extensive analysis of SVF and observed the presence of pericytes and supra-adventitial cells, in addition they documented the presence of endothelial progenitor cells (EPC) (Zimmerlin et al. 2012; Zimmerlin et al. 2009). Their finding confirmed those of Corselli and Crisan that pericytes and adventitial cells represent the 2 distinct MSC populations in SVF.
<table>
<thead>
<tr>
<th>CD Marker</th>
<th>+/-</th>
<th>Pericyte Reference</th>
<th>Adventitial Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD10</td>
<td>+</td>
<td>(Crisan et al. 2008)</td>
<td></td>
</tr>
<tr>
<td>CD13</td>
<td>+</td>
<td>(Crisan et al. 2008)</td>
<td></td>
</tr>
<tr>
<td>CD29</td>
<td>+</td>
<td>(Dar et al. 2012)</td>
<td></td>
</tr>
<tr>
<td>CD56</td>
<td>-</td>
<td>(Psaltis et al. 2010; Crisan et al. 2008)</td>
<td></td>
</tr>
<tr>
<td>CD106</td>
<td>-</td>
<td>(Zannettino et al. 2007)</td>
<td></td>
</tr>
<tr>
<td>CD133</td>
<td>-</td>
<td>(Crisan et al. 2008)</td>
<td></td>
</tr>
<tr>
<td>CD140</td>
<td>+</td>
<td>(Psaltis et al. 2010; Crisan et al. 2008; Dar et al. 2012; Tallone et al. 2011)</td>
<td></td>
</tr>
<tr>
<td>CD144</td>
<td>-</td>
<td>(Crisan et al. 2008)</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.6. Surface marker profile of pericytes and adventitial cells. 2 anatomically and phenotypically distinct populations of cells have been described that represent in vivo populations of MSC, namely pericytes and MSC. Whilst these 2 populations share common markers, differential expression of markers such as CD146 and αSMA allows distinction.

<table>
<thead>
<tr>
<th>Surface Marker</th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD166</td>
<td></td>
<td></td>
</tr>
<tr>
<td>αSMA</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>NG2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>STRO-1</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

1.7.8 Pericytes in development, repair and regeneration

A lack of understanding of the in vivo identity and location of MSC, and a lack of unique and specific markers had previously made their in vivo study difficult. In particular studies examining their role in development, repair and regeneration has been hampered by lack of adequate lineage tracing models. Our current understanding that the in vivo counterpart of MSC are perivascular cells has allowed pericyte specific lineage tracing models to be developed.

In vivo bone healing models have demonstrated the ability of transplanted pericytes to regenerate critical size calvarial defects in mice (James, Zara, Corselli, Askarinam, et al. 2012) and promote spinal fusion in rats (C. G. Chung et al. 2014). Recovery of hindlimb ischaemia has been demonstrated in murine models using pericytes derived from ES cells (Dar et al. 2012), and human blood vessels (Campagnolo et al. 2010). In murine models of
myocardial infarction, human muscle derived pericytes were injected intra-myocardially and demonstrated reduced fibrosis and enhanced functional recovery (Chen et al. 2012). These findings were supported by a subsequent study using human vein derived pericytes that demonstrated longterm improved functional recovery following transplantation (Katare & Madeddu 2013). In vitro myogenic differentiation, enhanced angiogenesis and muscle regeneration in vivo were seen in a murine model of muscle dystrophy when human placental derived pericytes were injected (Park et al. 2011).

Indirect evidence that pericytes may play a major role in development was observed by the spontaneous appearance of pericytes in cultures of embryoid bodies differentiating from human ES cells. In addition, these cells demonstrated MSC phenotype, function and the ability to rescue hind limb ischaemia in a murine model (Dar et al. 2012).

Tang et al demonstrated that pericytes were the native progenitors of white adipose tissue in murine adipose tissue with the use of a PPARγ (a central regulator of adipogenesis) reporter. Interestingly, whilst pericytes were found throughout the body, the cells responsible for adipogenesis were not found in tissues other than adipose tissue suggesting a tissue specific role (Tang et al. 2008).

Using NG2 as a pericyte marker, Feng et al used a NG2-Cre reporter mouse to demonstrate that vascular pericytes contribute to the regeneration of bone
following tooth injury (Feng et al. 2011). However they concluded that their contribution was not exclusive and other populations of cells were involved. Dellavalle et al utilised an Alkaline Phosphatase–Cre reporter mouse in a murine model of chemical induced skeletal muscle damage in which pericytes were shown to both differentiate into muscle fibres and also generate satellite cells (Dellavalle et al. 2011).

1.7.9 Pericytes and chondrogenesis

The long bones and the synovial joints are formed from condensations of the apical ectodermal ridge. Within the vascular channels of the developing foetal epiphyseal cartilage, pericytes are observed (H. C. Anderson et al. 2000). It was also observed that the vascular region of foetal menisci contained greater proportion of perivascular stem cells compared to the avascular part. When purified and grown in vitro these cells were capable of multi lineage differentiation and contributed to repair in a murine model of meniscal injury (Osawa et al. 2013).

Using an inducible pericyte reporter mouse, Matthews et al demonstrated that αSMA+ pericytes were responsible for a large proportion of the cells within the fracture callous (Matthews et al. 2014). The pericytes within the callous have also been shown to contribute to the chondroblasts and repair of the fracture (Brighton & Hunt 1997).
Studies that have described the ability of pericytes to function as multipotent MSC have demonstrated chondrogenesis of these cells in vitro (Crisan et al. 2008; Zannettino et al. 2007). The ability of microvascular pericytes to produce aggrecan (a key constituent of cartilage ECM), collagens and proteins associated with chondrogenesis has been observed in vitro (Diefenderfer & Brighton 2000; A E Canfield 2001), and has also been noted on histological examination of soft tissue wounds (Diaz-Flores, Gutierrez, Varela, et al. 1991). When transplanted in vivo, it was observed that bovine retinal pericytes gave rise to mature cartilage comprising cells embedded in an extracellular matrix rich in sulfated proteoglycans and type II collagen (Farrington-Rock 2004).

Early in vivo evidence that pericytes contribute to chondrogenesis was seen in a rabbit rib fracture model. 3 days following fracture, the proliferation, separation and migration of pericytes from the microvasculature was observed. Chondrogenic differentiation was observed by the presence of collagen fibrils and the production of matrix. In addition, mature chondrocytes retained elements of the basement lamina on their surface suggesting a vascular origin (Brighton & Hunt 1997). When perichondrium was transplanted in ectopic locations in mice, pericytes were seen to leave the vessel wall and contribute to neochondrogenesis (Diaz-Flores, Gutierrez, Gonzalez, et al. 1991).
In a related set of studies that examined the chondrogenic and chondroprotective function of subsets of MSC, it was observed that the CD146+ (pericyte) fraction showed enhanced function when compared to either CD146- or unsorted MSC. Hagmann et al compared CD146+ cells purified from human bone marrow derived MSC with unsorted MSC as controls. They observed that the CD146+ cells showed no difference in osteogenic or adipogenic ability when compared to controls. However, CD146+ cells showed increased amounts of GAG production following chondrogenic differentiation when compared to controls (Hagmann et al. 2014). When tested in a murine arthritis model, CD146+ cells showed reduction in the T-Helper cells associated with progression of disease and an increase in the levels of new cartilage produced in the diseased joint surface when compared to CD146- controls (Wu et al. 2016). In a further study, cells grown clonally from the human infrapatellar fat pad showed enhanced chondrogenesis and expression of the pericyte marker 3G5 (W. S. Khan et al. 2008).

Taken together these studies suggest a functional role for pericytes in the normal development of cartilage and the protection and repair of cartilage. In addition they contribute to neo-chondrogenesis in vivo, and also possess the ability to generate cartilage in vitro. They therefore have a justifiable potential in cartilage tissue engineering worthy of further examination.
1.8 Auricular tissue engineering

Surgeons and scientists have long recognised the need for a tissue engineered auricle to replace either the burden of harvesting costal cartilage, or the problems associated with alloplastic alternatives such as extrusion. Despite much research and early promise, a satisfactory solution still remains elusive.

The majority of strategies aimed at tissue engineering for auricular reconstruction consist of implantation of chondrocytes or stem/progenitor cells seeded on a scaffold. This is either implanted following a period of in vitro growth and/or differentiation, or implanted with the appropriate cues to promote in vivo maturation of the implant.

Early pioneering work by Cao and Vacanti used bovine articular chondrocytes seeded onto a synthetic, biodegradable polyglycolic acid (PGA) scaffold created in the shape of a 3 year old child's ear. Following implantation into nude mice for 12 weeks, mature neocartilage was observed that was able to retain the complex 3-dimensional shape of the ear. However during the 12 week process external splints were applied to maintain the shape and structure and when these were removed the detail and architecture was lost (Figure 1.10) (Cao et al. 1997). Although this study did not deliver a clinically relevant solution, it was fundamental in demonstrating
the role tissue engineering could play in solid tissue and organ growth with the appropriate choice of cells and scaffolds.

![Figure 1.10](image)

**Figure 1.10.** Photo taken from the original work of Cao and Vacanti demonstrating an ear shaped PGA scaffold seeded with bovine chondrocytes on the back of a mouse. Examination of constructs after 12 weeks demonstrated the presence of mature neochondrium with maintenance of 3-D architecture (Cao et al. 1997).

In selecting cells for auricular tissue engineering, scientists have explored the use of mature chondrocytes (articular, costal and auricular), and a variety of stem cells including perichondrium, bone marrow and adipose derived MSC (Jessop et al. 2016).

Chondrocytes have been shown to be able to produce neo-cartilage that resembles and functions like normal cartilage and is able to produce sufficient matrix to support 3-D structures (Cao et al. 1997). The application of chondrocytes is limited by the small amount of tissue able to be harvested without significant morbidity. This therefore requires cells to undergo serial expansion with a significant reduction in their chondrogenic phenotype with
increasing passage (C. Chung et al. 2006). However recent work has shown that with the addition of specific growth factors (bFGF) to the culture medium, auricular chondrocytes can be passaged to produce enough cells for auricular reconstruction without a significant reduction in their function from a small biopsy. These chondrocytes produced high quality cartilage that was rich in GAGs and elastin after 20 weeks in vivo implantation in a sheep model (Pomerantseva et al. 2016).

In the only reported clinical application of tissue engineered cartilage in ear reconstruction, Yanaga et al performed a 2 stage procedure where at the first stage cultured expanded auricular chondrocytes from the auricular remnant were injected into a pocket in the abdominal wall and left for 6 months. After this time, mature neo-cartilage had formed and was used in the 2nd stage to reconstruct the auricle. The authors report 2 - 5 year follow up with good retention of shape of the ear and no obvious signs of resorption (Yanaga et al. 2009).

Both bone marrow MSC and adipose derived MSC have been shown to be able to undergo chondrogenesis in vitro in numerous published studies (Kemmis et al. 2010; Jiang et al. 2010; Diekman et al. 2010; Jakobsen et al. 2009). The ease with which these cells can be harvested, and expanded with minimal change in their function makes them attractive candidates for use in tissue engineering. Despite these promising findings, the use of MSC in
auricular tissue engineering has been limited by the relatively small amounts of ECM produced (Shieh et al. 2004).

Variations in the chondrogenic capacity of MSC from different anatomical sites has also been demonstrated with bone marrow MSC showing greater cartilage production than adipose MSC in patient-matched controls (A. H. Huang et al. 2005). However these differences can be ameliorated with changes to the growth factors in the differentiation media with the addition of BMP-6 (Hennig et al. 2007; Diekman et al. 2009). However the addition of further growth factors is undesirable for clinical translation.

Recently, a population of highly clonogenic and chondrogenic CD44+, CD90+ perichondral stem cells were described. These cells were isolated from human auricular perichondrium and were capable of long term expansion without any obvious loss of function. When injected in to nude mice they were able to generate 2cm pieces of mature elastic cartilage with both perichondrium and chondrium that maintained its structure for over 10 months (Kobayashi et al. 2011). The minimal morbidity associated with harvesting perichondrium makes this source attractive, however the low yield of cells will require long periods of in vitro expansion prior to transplantation. Previous studies support the ability of chondroprogenitors derived from perichondrium to undergo serial expansion to the numbers required for auricular reconstruction whilst retaining their ability to differentiate in to chondrocytes and generate elastic cartilage (Yanaga et al. 2012; Van Osch
et al. 2000). *In vitro* expansion is less of a logistical problem in ear reconstruction as it is a staged, elective procedure and therefore could be planned around the required periods of *in vitro* growth. Furthermore the *in vivo* implantation in this model did not take into account the likely forces that would be placed on the structure in auricular reconstruction surgery.

As discussed in Chapter 1.7.9, pericytes have demonstrated the ability to undergo chondrogenesis *in vitro*, and produce GAGs and collagens when implanted *in vivo* (Diaz-Flores, Gutierrez, Gonzalez, et al. 1991; Farrington-Rock 2004). They have been shown to have a higher chondrogenic potential and chondroprotective function when compared to conventional MSC (Wu et al. 2016) (W. S. Khan et al. 2008).

<table>
<thead>
<tr>
<th>Cell source</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondrocytes</td>
<td>Able to produce high quality cartilage <em>in vivo</em> (Pomerantseva et al. 2016)</td>
<td>Limited donor site availability. Loss of function with <em>ex vivo</em> expansion (C. Chung et al. 2006) requires additional growth factors to prevent loss of function (Pomerantseva et al. 2016)</td>
</tr>
<tr>
<td>Bone marrow MSC</td>
<td>Large body of evidence to support ability of BM-MSC to differentiate into chondrocytes <em>in vitro and in vivo</em>.</td>
<td>Morbidity associated with harvest. Small quantities available. Production of ECM inadequate to support 3-D auricular scaffolds (Shieh et al. 2004)</td>
</tr>
</tbody>
</table>
Adipose derived MSC

Easy to harvest large volumes with minimal morbidity.

Lower chondrogenic potential when compared to other sources of MSC (A. H. Huang et al. 2005), requires additional growth factors to ameliorate this (Hennig et al. 2007).

Perichondrium derived stem cells

Able to generate mature elastic cartilage in vivo (Kobayashi et al. 2011), no loss of function with serial passaging (Yanaga et al. 2012; Van Osch et al. 2000).

Low yield rates. Long term in vivo function not assessed.

Pericytes

A pure and defined population that can be isolated from any tissue (Crisan et al. 2008). Greater chondrogenic potential when compared to other MSC (Hagmann et al. 2014). Shown to produce cartilage specific ECM in vitro and in vivo.

No previous examination of their ability to engineer cartilage in vivo and maintain 3-D structure.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipose derived MSC</td>
<td>Easy to harvest large volumes with minimal morbidity.</td>
<td>Lower chondrogenic potential when compared to other sources of MSC (A. H. Huang et al. 2005), requires additional growth factors to ameliorate this (Hennig et al. 2007).</td>
</tr>
<tr>
<td>Pericytes</td>
<td>A pure and defined population that can be isolated from any tissue (Crisan et al. 2008). Greater chondrogenic potential when compared to other MSC (Hagmann et al. 2014). Shown to produce cartilage specific ECM in vitro and in vivo.</td>
<td>No previous examination of their ability to engineer cartilage in vivo and maintain 3-D structure.</td>
</tr>
</tbody>
</table>

Table 1.7. Table detailing the advantages and disadvantages of different types of cells used in studies focused on auricular reconstruction.

In addition to different cell types, numerous different biomaterials have been examined for their ability to support chondrogenesis and act as scaffolds for tissue engineering (Table 1.9). One of the most common groups of synthetic biomaterials tested are the aliphatic polyesters such as polyglycolic acid (PGA), polylactic acid (PLA) and polycaprolactone (PCL). These polymers have demonstrated the ability to support cell growth, differentiation and
production of cartilage ECM (Cao et al. 1997; Mahmoudifar & Doran 2010; Valonen et al. 2010). They are biodegradable through hydrolysis of the ester linkages, however they produce acidic by-products which can cause local tissue reaction and also degradation of the engineered tissue (Puppi et al. 2010). In a rabbit model both PCL and PLA constructs were tested and lacked sufficient structural integrity to overcome the forces applied to them resulting in the detail and architecture of the 3-d structure being lost (Shieh et al. 2004). Furthermore, the degradation products of aliphatic polyesters have demonstrated antigenicity when implanted in immunocompetent animals (Nayyer et al. 2012).

Naturally occurring biomaterials such as silk, chitosan, alginate and hyaluronic acid have also been used in cartilage tissue engineering (Awad et al. 2004; Wang et al. 2005). Whilst these naturally occurring biomaterials have demonstrated good bioactivity and ability to support chondrogenesis, problems with their long-term structural properties to maintain complex 3-D structures have been raised and require further long-term in vivo assessment (Sterodimas et al. 2009).

Whilst these biomaterials have been used extensively, the use of natural products should be done with caution, especially in clinical application. Products derived from natural sources can show significant batch to batch variation due to inherent biodiversity and as such are poorly defined. Any product/cell that is intended for clinical use must have all elements defined
for both consistency and traceability (EU directives 2003/94/EC and 2004/24/EC). In addition to this, those derived from animal products can induce immune responses and risk contamination with pathogens (Martin et al. 2005).

Synthetic biomaterials offer potential advantages over natural alternatives by eliminating batch to batch variability and any risk of infection or immunogenicity. In addition to this they offer much greater tunability such as molecular weight, cross-linking, chemical composition, addition of growth factors, mechanical strength and degradability. The ability to consistently predict, control and reproduce these factors are especially advantageous for synthetic biomaterials as a construct for tissue engineering in clinical applications. This is particularly relevant to auricular engineering when much of the long term failure of previous biomaterials has related to unpredictable degradation and the inability to maintain structural integrity (F. Khan et al. 2009).
<table>
<thead>
<tr>
<th>Natural</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Full, rapid and natural degradation (this is not always beneficial when long term support is needed eg bone)</td>
<td>• Immunogenicity</td>
</tr>
<tr>
<td>• Can function at a molecular level not just macroscopic</td>
<td>• Potential for transmission of pathogens</td>
</tr>
<tr>
<td></td>
<td>• Lot to lot variability</td>
</tr>
<tr>
<td></td>
<td>• Poor biomechanical properties</td>
</tr>
<tr>
<td></td>
<td>• Limited tunability</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Synthetic</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>• Minimal lot to lot variation</td>
<td>• Toxicity</td>
</tr>
<tr>
<td>• Mechanical and chemical properties easily altered</td>
<td>• Chronic inflammation</td>
</tr>
<tr>
<td>• Cheap and easily scalable</td>
<td>• Biocompatibility hard to predict and must be tested</td>
</tr>
<tr>
<td>• Many currently in clinical use with FDA approval</td>
<td>• Incomplete degradation with harmful degradation products</td>
</tr>
<tr>
<td>• Easy to combine and blend with other materials</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.8: Advantages and disadvantages of natural and synthetic biomaterials

Recent developments in 3-D printing have allowed researchers to use imaging systems to design and print individual auricular shape scaffolds. Using computer-aided design, a CT scan of an ear was used to produce a PLA/PGA woven scaffold in the shape of a human ear. The scaffold was seeded with articular chondrocytes and after 12 weeks of *in vitro* growth showed mature cartilage and good mechanical strength (Y. Liu et al. 2010). Whilst this study showed promising results, 12 weeks of *in vitro* growth is not sufficient to determine of the biomaterials will retain sufficient integrity for long term *in vivo* use.
<table>
<thead>
<tr>
<th>Biomaterial</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silicone/silastic</td>
<td>Cheap and easy to manufacture.</td>
<td>High rates of extrusion and infection mean its use has been abandoned (Cronin 1966).</td>
</tr>
<tr>
<td>Medpore</td>
<td>Reproducible charactersitics.</td>
<td>High rates of extrusion, infection and fracture (Romo &amp; Reitzen 2008).</td>
</tr>
<tr>
<td>POSS-PCU</td>
<td>Porous structure promotes in growth of fibroblasts and blood vessels (Nayyer et al. 2014). Has shown ability to support chondrogenesis of ADSC (Guasti et al. 2014). Biophysical properties similar to native elastic cartilage (Nayyer et al. 2014)</td>
<td>No long term in vivo data. Not biodegradable so may encounter same problems of extrusion as medpore</td>
</tr>
<tr>
<td>PLA/PGA/PCL</td>
<td>Able to support growth and chondrogenesis of chondrocytes and also MSC populations. Can be manufactured in many different ways including 3-D printing (Y. Liu et al. 2010)</td>
<td>Unable to maintain structure in long term in vivo studies. Acidic degradation products damaging to cartilage (Shieh et al. 2004)</td>
</tr>
<tr>
<td>HA</td>
<td>Highly biocompatible and naturally occurring component of ECM. Has shown ability to support chondrogenesis</td>
<td>Heterogeneity of cartilage within constructs. No data on structural ability of HA to maintain 3-D architecture</td>
</tr>
</tbody>
</table>

Table 1.9. Table detailing the advantages and disadvantages of different types of biomaterials used in studies focused on auricular reconstruction.
1.9 Summary

Plastic and reconstructive surgery is a surgical discipline that aims to restore form and function to patients. One of the major limiting factors in the ability of surgeons to reconstruct defects is adequate donor tissue that can be used without significant additional morbidity to the patient.

In the case of ear reconstruction, the current gold standard tissue to replace the deficient cartilage of the ear is costal cartilage due to both the quantity required, and the necessary biomechanical properties. The burden of harvesting ribs for this purpose is significant and results in prolonged periods of in patient stay in hospital and can result in adverse scarring, chest deformity, pneumothorax and spinal deformity. It has therefore been long recognized that an alternative to costal cartilage would be advantageous.

Synthetic structures have been used in ear reconstruction including silastic and Medpor® (porous poly ethylene), however their use has failed to gather widespread support due to high rates of infection, extrusion and fracture of the implants. Tissue engineering is an appealing alternative that could potentially deliver an autologous implant with the required biomechanical properties with minimal morbidity to the patient. The ultimate aim of engineering a suitable replacement for auricular reconstruction will require a cell source capable of chondrogenesis and producing sufficient cartilage ECM (GAGs and cartilage specific collagens) to maintain 3-D structure and detail whilst overcoming the external forces applied to the ear. In addition, a
suitable scaffold will be required to promote chondrogenesis and provide the structural and biomechanical support required whilst the neocartilage develops.

Previous work on auricular engineering has used a variety of cell types and biomaterials. Chondrocytes have shown the ability to produce large amounts of neocartilage and ECM, however limited donor sites and the requirement for extended periods of *in vitro* growth to generate sufficient numbers have limited their clinical application. Conversely, MSC can be derived from multiple readily accessible sources and have shown the ability to undergo extended periods of *in vitro* growth with minimal reduction in their function. However, longer term *in vivo* studies have demonstrated that MSC produce insufficient amounts of ECM to support 3-dimensional structures. Pericytes have recently emerged as candidates for cartilage tissue engineering. They are present in all vascularized tissues and represent the *in vivo* precursor of the MSC observed *in vitro*. Studies have demonstrated the ability of pericytes to undergo chondrogenesis and produce cartilage specific ECM *in vitro* and *in vivo*. Comparative studies examining the chondrogenic potential of pericytes have shown enhanced chondrogenic function when compared to conventional MSC.

The most widely investigated biomaterials in auricular engineering are the aliphatic polyesters such as polyglycolic acid (PGA), polylactic acid (PLA) and polycaprolactone (PCL). They have demonstrated the ability to support
the growth of chondrocytes and stem cell populations both in vitro and in vivo, and the production of ECM. However, the degradation rates and acidic products of degradation have resulted in loss of architecture and mechanical stability in longer term in vivo studies. Therefore identification of novel polymers with the required biological and biomechanical properties are fundamental to the successful generation of a tissue engineered auricle.

1.10 Thesis aims

The aim of this thesis is to identify cells and polymer based substrates that can be used for human auricular tissue engineering. To do this, the thesis has been structured with the following general aims. Further details of the specific aims are contained within the relevant chapter.

Tissue procurement (Chapter 3)

To establish a working biobank of human adipose tissue and adipose derived cells with all the appropriate ethical approval for subsequent research.

Cell characterisation and isolation (Chapter 4)

To perform a detailed analysis and characterisation of the identity, location and frequency of pericytes within adipose tissue, and to develop methods for their isolation and purification focused on the delivery of healthy cells for clinical use.
**Polymer identification and analysis (Chapter 5)**

To use high-throughput polymer microarray screening to identify potential substrates with high binding affinity for adipose derived pericytes. To examine and quantify the effect that these polymers have on the functions of pericytes with relevance to tissue engineering.

Taken together these achievements would represent a clear advance in the steps towards the clinical translation of auricular tissue engineering.
2 Materials and methods

2.1 Tissue collection and processing

Subcutaneous adipose tissue was collected from healthy adult volunteer donors undergoing cosmetic lipectomy procedures with prior written consent. A small number of samples were also collected from patients undergoing breast reconstruction surgery. Ethical approval for the collection, processing, storage and subsequent research was granted in November 2010 by South East Scotland Research Ethics Committee 03 (SESREC03). For full details of the process please see Chapter 3. Tissue was collected immediately after surgical removal and stored in sterile containers at 4°C until required for the preparation of SVF.

2.1.1 Preparation of SVF

Whole Fat

Incisions were made using a scalpel in the adipose tissue to divide the Scarpa’s fascia and expose the superficial adipose tissue. Adipose tissue was mechanically minced prior to enzymatic digestion using a standard cheese grater that was cleaned with 70% IMS prior to use. Approximately 150cc of grated adipose tissue was mixed with equal volumes of PBS and transferred to 50ml ‘Falcon’ centrifuge tubes (Corning – 430828) and shaken vigorously for 30 seconds. Tubes were centrifuged at 1800rpm for 10 minutes. After spinning, the tissue separated into 3 phases, oil at the top, fat
in the middle and blood and liquid at the bottom. The top and bottom phases were removed and 100cc of the washed fat was transferred to a large container.

**Lipoaspirate**

50cc of lipoaspirate was transferred into each of 4 x 50ml ‘Falcon’ centrifuge tubes (Corning – 430828) and then centrifuged at 1800rpm for 10 minutes. After spinning, the top and bottom layers were discarded, and the fat transferred to 4 fresh 50ml centrifuge tubes and made up to a total volume of 50cc in each tube with PBS. Tubes were shaken vigorously for 30 seconds and then centrifuged at 1800rpm for 10 minutes. The top and bottom phases are removed and a total of 100cc of washed fat was transferred to a large container.

**Enzymatic digestion**

100ml of digestion solution (DMEM + 3.5% BSA + 1mg/ml type II collagenase) was added to the fat and transferred to the shaking water bath at 37°C 250rpm for 40 minutes. After 40 minutes the reaction was quenched by adding 40ml of FBS (Gibco, 10270-106). 50ml of the solution was then transferred to each of 4 50ml falcon tubes and centrifuged at 1800rpm for 10 minutes. The supernatant (containing oily fat and adipocytes) was aspirated and the pellets resuspended in 25ml of PBS-5mM EDTA. Clumps were disrupted as much as possible by pipetting up and down and any persistent
clumps discarded. The solution was then successively filtered through 100µm and 70µm cell strainers (Fisherbrand, 22363547). The filtered solution was made up to a total volume of 50cc with PBS-5mM EDTA and centrifuged at 1500rpm for 10 minutes. Following centrifugation, the supernatant was aspirated and discarded and the pellet resuspended in 10ml of RBC lysis buffer and left at room temperature for 5-10 minutes. Red cell lysis buffer was made from 2 stock solutions (stock 1 = 8.3g ammonium chloride in 1000ml water, and stock 2 of 20.59g Tris base in 1000ml water after adjusting pH to 7.65 with 1M hydrochloric acid). The final solution contains 9 parts of stock 1, and 1 part of stock 2 and the pH is adjusted pH to 7.65 with 1M hydrochloric acid.

20ml of PBS-5mM EDTA was added to the tube and the resulting suspension filtered through a 40µm cell strainer. The filtrate was made up to a total volume of 50ml with PBS-5mM EDTA and centrifuge at 1500 rpm for 10 minutes. The supernatant was aspirated and discarded and the pellet (comprised of the SVF) was resuspended in 1ml of 2% FBS in PBS for counting and immunostaining.

2.2 Flow cytometry

2.2.1 Immunostaining of fresh adipose tissue for FACS

Samples resuspended in 1ml of 2% FBS in PBS were stained for 20 mins in the dark on ice using the following antibodies and concentrations:
Table 2.1. Antibodies and the concentrations used for FACS of adipose derived pericytes.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31 PE (BD Biosciences – 555446)</td>
<td>1:100</td>
</tr>
<tr>
<td>CD34 FITC (BD Biosciences – 555821)</td>
<td>1:100</td>
</tr>
<tr>
<td>CD45 APC Cy7 (BD Biosciences – 557748)</td>
<td>1:100</td>
</tr>
<tr>
<td>CD146 Alexa 647 (AdB Serotec - MCA2141A647)</td>
<td>1:100</td>
</tr>
</tbody>
</table>

Excess antibody was washed off by adding 3ml of PBS + 2% FBS and centrifuging for 5 minutes @ 1200rpm before being resuspended in PBS + 2% FBS for FACS.

To allow for appropriate set up of the flow cytometer, a sample with no stains added was collected. Samples were also stained in identical manner with matching isotype controls with all stains in a single sample - PE Mouse IgG κ Isotype Control (BD Biosciences, 555749), FITC Mouse IgG1 κ Isotype Control (BD Biosciences, 555748), APC Cy7 κ Isotype Control (BD Biosciences, 557872), Alexa 647 Mouse IgG1 Negative Control (AdB Serotec, MCA928A647). Compensation beads (BD Biosciences – 552843) were stained with each of the individual antibodies to allow for any spillover in the emission spectra of the fluorochromes to be detected and compensated for.
2.2.2 Analysis of cultured cells by flow cytometry

Cultured cells were washed with PBS and then covered with an appropriate amount of 0.25% trypsin (Gibco, 25200-072) (12 well plate = 100µl / well, T75 flask = 1ml) and placed in the incubator for 5 minutes to detach the cells. 20 times the volume of SGM was added to each well to quench the action of the trypsin. The solution was then transferred to a 15ml or 50ml centrifuge tube and centrifuged at 1000rpm for 5 minutes. The supernatant was aspirated and the cells resuspended in an appropriate volume of 2% FBS in PBS for immunostaining.

Multicolour panels of antibodies were established based on available fluorochromes for pericyte and MSC markers. All antibodies were used at a concentration of 1:100, and samples were stained for 20 minutes on ice in the dark. Excess antibody was washed off by adding 3ml of 2% FBS in PBS and centrifuging for 5 minutes @ 1200rpm before being resuspended in 2% FBS in PBS for analysis. In addition to the stained samples, matched isotype controls, individual compensation beads and an unstained sample were all processed.

2.3 Long term culture of pericytes

The Standard Growth Medium (SGM) used in the culture of pericytes was DMEM + Glutamax (Gibco, 61965-026), with 20% FBS (Gibco, 10270-106) and 1% Penicillin / Streptomycin (Sigma, P4333).
**Passage 0:** For the first passage, cells were cultured on gelatin coated plates. To prepare the plates, 200µl of 0.1% gelatin (Sigma - G1393) was added to each well of a 12 well plate and left for 10 minutes at 4°C before excess gelatin was aspirated and discarded. Immediately following sorting, the pericyte containing solution was centrifuged at 1500rpm for 5 minutes. The supernatant was aspirated and discarded and the cells resuspended in EGM2 (Lonza - CC-3162) to give a final concentration of 20000 cells/ml. The cell containing solution was pipetted to the gelatin coated 12 well plates at a density of 40000 cells/cm² and transferred to a humidified incubator. Media was changed every 5 days until cells reached confluency.

**Passage 1:** When confluent, wells were washed with PBS and then covered with 100µl of 0.25% trypsin and placed in the incubator for 5 minutes to detach the cells. 2ml of SGM was added to each well to quench the action of the trypsin. The solution was then transferred to a 15/50ml centrifuge tube and centrifuged at 1000rpm for 5 minutes. The supernatant was aspirated and the cells resuspended in an appropriate volume of SGM. Cells were split in a 1:6 ratio and transferred to uncoated cultureware. 24 hours after plating, the media was aspirated and discarded to remove and dead and non-adherent cells. Cells were washed with PBS and re-covered with SGM which was changed 3 times per week until confluent.
**Passage 2 onwards:** For all subsequent passages cells were split as above and replated at a ratio of 1:6 and cultured in SGM.

### 2.3.1 Cryopreservation of PSC

Cells were stored for subsequent experiments in frozen cryovials with one confluent T75 flask per cryovial. Cells cultured in T75 flasks had media aspirated and were rinsed with PBS. 1ml of 0.25% trypsin was added to each flask and placed in an incubator for 5 minutes. 10ml of SGM was added to each flask and the cell containing solution transferred to 50ml falcon tubes and centrifuged at 1200rpm for 5 minutes. Supernatant was aspirated and discarded and the pellet was resuspended in 1ml of freezing media (FBS + 10% EDTA) for each T75 flask of cells. The freezing media and cells were vortexed to ensure the cells were equally distributed and 1ml of solution was transferred to a labeled 1.5ml cryovial. Cryovials were placed in the wells of a Mr Frosty™ (Thermo Scientific) freezing container and placed in a -80°C freezer for a minimum of 24 hours. Cells stored for less than 3 months could then be transferred to a standard container within the -80°C freezer, those to be stored for periods greater than 3 months were transferred to liquid nitrogen storage tanks.

**Thawing of cryopreserved PSC**

When required, cells were retrieved from the -80°C freezer or liquid nitrogen and transported to the tissue culture laboratory on dry ice. Cryovials were
rapidly thawed at 37°C in a waterbath and the solution of freezing media and cells transferred to a 50ml falcon tube and resuspended in an appropriate volume SGM. Depending on requirements, 1 cryovial was replated in a ratio of 1:1 to 1:4 in T75 flasks.

2.4 Immunohistochemistry of adipose tissue

Fresh whole subcutaneous adipose tissue was cut into pieces of approximately 1cm³. The pieces of adipose tissue were washed thoroughly in PBS to remove blood and then fixed overnight in 4% paraformaldehyde (PFA) at 4°C.

Samples were removed from PFA, washed 3 times in PBS and immersed in solutions of increasing concentrations of sucrose in PBS at 4°C. 10% sucrose for 1 hour, followed by 20% sucrose for 1 hour, followed by 30% sucrose overnight.

Samples were removed from sucrose and placed in OCT containing foil molds and allowed to soak for 20-30 minutes. Samples were then freeze dried on dry ice and stored at -80°C until required. Frozen human adipose tissue was cut in 10µm section using a cryostat at -50°C and mounted on Superfrost™ Plus microscope slides (Fisher Scientific).

Day one: The slides with the adipose tissue were taken out of the -80°C freezer and left at room temperature for 30mins to air dry. Sections were then
fixed in a solution of ice cold Methanol/Acetone (50:50) for 10 minutes. Slides were then washed in PBS/Tween 20, three times for five minutes each. Sections of the slides without specimen on were dried using a tissue and then the sections were drawn around using a wax pen. Sections were blocked with protein block (Dako) and incubated for one hour at room temperature. Slides were then washed with PBS/Tween20, three times for 5 min each. Slides were then incubated overnight at 4°C with primary antibodies (Table 2.1) diluted at 1:100 in antibody diluent (Dako). Typically 100µl of antibody containing solution was added to each slide, however this would vary depending on the size of the sample.

Second day: Slides were washed with PBS/Tween20 three times for 5 minutes, to remove the primary antibody. Secondary antibodies (goat-anti-rabbit Alexa Fluor 546/ goat-anti-rabbit Alexa Fluor 488/ goat-anti-mouse Alexa Fluor 488) diluted at 1:600 in antibody diluent were then added in equal volumes to the primary antibody. The secondary antibodies were incubated for 1 hour at room temperature in the dark. After incubation, samples were washed with PBS/Tween20 three times for five minutes each. For co-staining with unconjugated second antibodies, the previous stages were repeated. For co-staining with conjugated secondary antibodies, samples were blocked with protein block (Dako) at room temperature for one hour. Subsequently conjugated secondary antibodies diluted at 1:100 in antibody diluent (Dako) were added and incubated at RT for 2 hours. After incubation, slides were washed with PBS/Tween20 three times for 5 minutes each followed. Finally, Fluorescent Media with DAPI counterstain was
mounted and the slides were cover slipped. Samples were visualized using an upright widefield Olympus BX61 microscope.

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Supplier</th>
<th>Catalog No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>αSMA</td>
<td>Sigma-Aldrich, St. Louis, MO, USA</td>
<td>F3777</td>
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<tr>
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</table>

Table 2.2: Antibodies used for immunostaining of adipose tissue and pericytes.

2.5 Immunocytochemistry of cultured cells

Cells were cultured to desired confluence in 12 well plates, or on standard glass coverslips and placed in 12 well plates. Wells were washed twice in
PBS and cells were then fixed in ice cold methanol for 10 minutes before being washed twice again in PBS. Blocking was performed for 30 minutes with protein block (Dako), followed by a single wash with PBS/Tween20. Finally, the cells were covered with the appropriate primary antibody. 100µl of the antibody diluted 1:100 with antibody diluent (Dako) was added to each well. For the negative controls, only PBS was added to the well in place of a primary antibody. Plates were then covered with foil and incubated overnight on a rocking platform in a cold-room (4°C).

The next day, the wells were washed for 5 minutes, three times in PBS-Tween20 in a rotating plate. For primary conjugated antibodies, wells were allowed to air dry before 1 drop of DAPI-containing mountant was added and then coverslips placed in each well and left to dry in the dark. For unconjugated primary antibodies, each well was treated with a secondary antibody for one hour at room temperature in the dark. The secondary antibodies were made up at a 1:400 in antibody diluent. After one hour, wells were washed for 5 minutes, three times in PBS. Wells were left to air dry before one drop of DAPI mounting medium was added to each well and a small coverslip was dropped into the wells. To make sure that there were no bubbles, coverslips were pressed lightly. The samples were imaged by a fluorescence microscope (Olympus BX61).
2.6 Differentiation protocols and quantification

As detailed in the criteria described by the ISCT to define MSC, cells must be able to demonstrate differentiation to adipocytes, osteoblasts and chondrocytes in vitro (Dominici et al. 2006). Many different protocols to do this have been published, but all rely on the addition of a variety of growth factors to media. In addition, many ‘off the shelf’ differentiation kits are available. To establish that cells had differentiated, functional stains were performed as detailed below. In addition, and where possible, quantification of differentiation was performed as described below.

Pericytes were grown in T75 flasks in SGM until sufficient numbers of cells were obtained. Flasks had media aspirated and were rinsed with PBS. 1ml of 0.25% trypsin was added to each flask and placed in an incubator for 5 minutes. 20mls of SGM was added to each flask and the cell containing solution transferred to 50ml falcon tubes and centrifuged at 1200rpm for 5 minutes.

2.6.1 Adipogenesis

Cells were then transferred to 12 well plates with 20,000 cells in each well in 2ml of SGM. Cells were then placed in an incubator and allowed to attach and grow to 40% confluency (approximately 48hours). Media was aspirated and then rinsed with PBS. 1ml of adipogenic media (StemPro® Adipogenesis
Differentiation Kit, Gibco) was added to each well and plates returned to the incubator. Media was changed 3 times per week for a total of 3 weeks. Controls were performed by using SGM instead of adipogenic media.

Assessment of adipogenesis - Oil red O staining
Wells were washed four times with PBS. 4% PFA was added to the wells and incubated at 37°C for 10mins, before washing four times with ddH₂O. ddH₂O was aspirated and 60% isopropanol was added for 5 minutes at room temperature. Wells were then dried without washing, before adding 1ml of Oil red O solution and incubating at room temperature for 10 minutes (Working solution made by adding 6mls of oil red O stock to 4 mls distilled water). Wells were then washed with ddH₂O four times.

Quantification of adipogenesis
Adipogenesis was quantified using an Oil Red O elution assay (Chemicon® International – ECM950). Following 3 weeks of differentiation in adipogenic growth media cells were fixed and stained with Oil Red O as described above. Following this, 0.5ml of Dye Extraction Solution was added to each well and transferred to an orbital shaker for 15 minutes. 150µl of the solution was then transferred in triplicate to the wells of a 96 well plate and quantified using colourimetric detection at 520nm.
2.6.2 Osteogenesis

Cells were transferred to 12 well plates with 10,000 cells in each well in 2ml of SGM. Cells were then placed in an incubator and allowed to attach and grow to 25% confluency (approximately 48 hours). Media was aspirated and then rinsed with PBS. 1ml of osteogenic media (StemPro® Osteogenesis Differentiation Kit, Gibco) was added to each well and plates returned to the incubator. Media was changed 3 times per week for a total of 3 weeks. Controls were performed by using SGM instead of osteogenic media.

Assessment of osteogenesis - Alizarin Red staining

Wells were washed four times with PBS, and then 4% PFA was added and incubated at room temperature for 30 minutes. Wells were then washed four times with ddH20, after the ddH2O was aspirated 1ml of alizarin red solution added to each well for 30 minutes. Wells were then washed with ddH2O four times. Plates could either be processed/imaged immediately or stored at -20°C until required.

Quantification

800µl of 10% (v/v) acetic acid was added to each well and incubated for 30 minutes at room temperature on a rotating platform. The loosely attached monolayer of cells was removed with cell scraper if necessary and transferred with the 10% acetic acid to a 1.5ml microcentrifuge/eppendorf tube and vortexed for 30 secs. The solution was then heated to 85°C for 10
minutes, before being transferred to ice for 5 minutes. It was then centrifuged at 20,000g for 15 minutes, before 500µl of the supernatant was transferred to a new 1.5ml microcentrifuge tube. 200µl of 10% (v/v) ammonium hydroxide was added to neutralize the acid. 150µl aliquots of the supernatant were transferred in triplicate (or more if required) into the wells of a 96 well plate. Readings were made at 405nm on a light reader.

2.6.3 Chondrogenesis

Monolayer
Cells were then transferred to 12 well plates with 20,000 cells in each well in 2ml of SGM. Cells were then placed in an incubator and allowed to attach and grow to 50% confluency (approximately 48hours). Media was aspirated and then rinsed with PBS. 1ml of chondrogenic media (StemPro® Chondrogenesis Differentiation Kit, Gibco) was added to each well and plates returned to the incubator. Media was changed 3 times per week for a total of 4 weeks. Controls were performed by using SGM instead of chondrogenic media.

3-D culture (pellet)
Solution of SGM containing 100,000 cells/ml was prepared and 3ml added to a 15ml centrifuge tube. Tubes were centrifuged at 300g for 3 minutes and then transferred to the incubator. Over 48 hours the cells condensed to a pellet that was freed from the sides/bottom of the tube by gentle agitation.
SGM was carefully aspirated to avoid aspiration of the pellet and replaced with 1ml of chondrogenic media (StemPro® Chondrogenesis Differentiation Kit, Gibco). Media was changed 3 times per week for a total of 4 weeks and at each change the pellet was freed from the bottom of the tube. Controls were performed by using SGM instead of chondrogenic media.

Assessment of chondrogenesis - Alcian Blue staining

Pellets were removed from the centrifuge tubes and air dried for 20 minutes before being placed in the bottom of OCT containing foil molds. Samples were then freeze dried on dry ice and stored at -80°C until required. Frozen pellets was cut in 10µm sections using a cryostat at -50° C and mounted on Superfrost™ Plus microscope slides (Fisher Scientific).

Wells/slides were washed four times with PBS and then 4% PFA was added and incubated at room temperature for 10 mins. Wells/slides were washed four times with ddH$_2$O, and then Alcian Blue solution (1% Alcian Blue in 1% Acetic acid) was added to each well/slide for 15 minutes (1ml per well, 100 µl per section). Wells/slides were washed with ddH$_2$O four times.

Quantification of chondrogenesis (monolayer)

0.5ml of 1M HCl was added to each well and placed on a rotating plate for 30 minutes. 150µl aliquots of the supernatant were transferred in triplicate (or more if required) into the wells of a 96 well plate. Readings were made at 620nm on a light reader.
2.7 Polymers

The polyacrylate/acrylamide library was synthesised by Dr. Mizomoto, and the polyurethane library was synthesised by Dr. Thaburat under the supervision of Professor Mark Bradley (University of Edinburgh).

2.7.1 Preparation of agarose coated slides

Glass slides were placed in to 1M NaOH for 4 hours and then rinsed with distilled water followed by 100% acetone. Glass slides were then air dried before being immersed in an acetonitrile solution of 2-amine ethyl trimethoxysilane (1% wt/v) for 1 hour. The glass slides were then washed with acetone and placed face down on an aluminium foil-lined tray in a vacuum oven at 100°C for 2 hours. The glass slides were then dip-coated in 1% agarose aqueous solution at 60°C. Agarose was wiped from the underside and the agarose coated glass slides were air dried for 24 hours before being used for printing.

2.7.2 Printing of microarrays

Polymers were contact printed onto agarose coated slides using a Q-arrayer\textsuperscript{mini} (Genetix, UK), using aQu solid tipped pins (K2785, Genetix, UK) inked from a 384 well plate containing 1% (w/v) polymer solutions in N-methylpyrrolidone (NMP). Each spot was stamped 5 times with 200ms
inking time and 100ms stamping time resulting in spots of an approximate diameter of 310µm. For the initial screen polymers were printed in quadruplicate in a 8 x 48 pattern with 2 empty areas resulting in 1536 dots of 382 discreet polymers. Slides were dried in a vacuum oven at 40 °C overnight.

2.7.3 Screening of microarrays

Each polymer spot was printed on the slide in quadruplicate to act as an internal control, and each sample was screened in duplicate to act as an external control. The polymer microarray screen was performed with 4 separate patient samples (n=4), and flow cytometry prior to use assessed the purity of each sample. Slides were placed in 4 well slide trays and sterilised for 30 minutes under UV light. Pericytes were suspended in SGM at a concentration of 100,000 cells/ml, and 5ml of the cell containing media was placed on each slide. Slides were then placed in an incubator (37°C + 5% O₂) for 24 hours. At the end of the incubation, slides were washed in PBS for 5 minutes on a rotating plate to remove any non-adherent cells or loosely attached cells. Cells were fixed by immersion in ice cold methanol for 10 minutes then rinsed in PBS for 5 minutes before being left to air dry. Coverslips were attached using DAPI containing mountant (Vectashield, Vector Laboratories). Analysis of the binding of cells to the polymer spots was performed using a high resolution, high content screening (HCS) platform equipped with a Nikon 50i fluorescence microscope (x20 objective)
with a motorised X-Y-Z stage. The automated scanning platform is programmed to capture multiple channel (DAPI, FITC, bright field) high resolution images for each spot at defined intervals based on the spatial arrangements of the polymer spots on the microscope slide. The microscope is equipped with Pathfinder™ software that performs automated analysis of the captured images allowing rapid analysis of multiple parameters including cells number, shape, size and fluorescence intensities.

Data was analysed using Graphpad software. Overall differences between all groups were calculated using Kruskall-Wallis test, and differences between individual groups and controls were calculated using Dunnett’s multiple comparison test (One way ANOVA), which allows multiple groups to be compared to control.

2.7.4 Spin coating

22mm diameter glass coverslips (Menzel-Gläser, Germany) were cleaned with tetrahydrofuran (THF). 60µl of each of the polymer solutions (PA133, 136, 337 and 338 in 2.0% w/v in THF, PA210 2.0% w/v in NMP) was placed onto the coverslips and spin coated for 2 seconds at 2000 rpm using spincoater Model P6700 Series (Specialty Coating Systems, Inc). Coverslips were dried in a vacuum oven overnight at 45 °C / 200 mbar, and irradiated with UV light for 20 minutes prior to use.
3 Analysis of patients attitudes towards the donation of adipose tissue for medical research, and the establishment of a research tissue bank (RTB)

Parts of this chapter were previously published in:

West CC, Murray IR, González Z, Hindle P, Hay D, Stewart KJ, Péault B


Ethical, Legal and Practical Issues of Establishing an Adipose Stem Cell Bank for Research.


Patient’s Attitude towards the Donation and Use of Adipose Tissue and Adipose Derived Stem Cells for Research.
3.1 Introduction

In this chapter the legal, ethical and practical issues relating to the banking of human adipose tissue for research in the UK are examined, with reference to international guidelines and regulations. An analysis of the perceptions of patients towards donating tissue was performed using a questionnaire aimed to help with the design of appropriate information sheets and tailor the application to the needs of the researchers without compromising the expectations of the patients. The experiences of establishing a research tissue bank (RTB) are shared, including the necessary infrastructure and the submission of an application to a Research Ethics Committee (REC).

Stem cells are central to regenerative medicine, and understanding these cells is fundamental to developing new treatments. One of the challenges facing regenerative medicine is finding suitable, safe and ethical sources of stem cells for both research and therapy. Native stem cells come in many forms from the totipotent and pluripotent cells found in the morula and blastocyst respectively, through to multipotent, bipotent and unipotent stem cells found in adult organisms (Kørbling & Estrov 2003). Embryonic Stem Cells (ES) are pluripotent cells derived from the inner cell mass of early embryos, and offer huge promise in regenerative medicine. However their collection, storage and use raises many ethical concerns. In addition, their application is more strictly regulated in comparison to adult stem cells such as mesenchymal stem cells (MSC). MSC are multipotent cells that are capable of differentiating into bone, cartilage, fat, muscle and tendon (Caplan
In addition, MSC secrete many bioactive molecules that are immunomodulatory, anti-apoptotic, anti-fibrotic, angiogenic and mitotic therefore suggesting a general regenerative function not just limited to differentiation into cells of a mesenchymal lineage (Caplan & Correa 2011). The discovery that these cells can be found in almost all postnatal tissues is of particular interest in their clinical application as it means that cells that are broadly identical in their function and phenotype can be isolated from redundant tissue such as placenta or deciduous teeth, or from dispensable sources such as adipose tissue.

Access to human tissue is critical to medical research, however the laws and regulations surrounding gaining ethical and legal access to tissue are often poorly understood. Recently, there has been a huge increase in the interest surrounding the therapeutic application of adipose tissue, and adipose derived stem cells. Adipose tissue is easily accessible and readily abundant even in individuals of healthy weight and therefore offers an excellent source of stem cells (Gimble et al. 2007). As plastic surgeons routinely remove large volumes of adipose tissue through reconstructive and cosmetic procedures, this has resulted in many surgeons being either directly or indirectly involved in research using adipose-derived stem/stromal cells (ASC). In addition to ASC, adipose tissue is also an excellent source of adipocytes, endothelial cells and vascular smooth muscle cells. In cases where there is excision of redundant skin there are also abundant dermal and epidermal cells.
The chondrogenic potential of ASC has been demonstrated in vitro and in vivo (Diekman et al. 2010; Betre et al. 2006; Awad et al. 2004). Their ability to generate chondrocytes has been shown to be equal to bone marrow derived MSC with minor alterations to the differentiation protocols (Hennig et al. 2007). In addition, subsets of stem cells within adipose tissue with highly chondrogenic potential have been identified (W. S. Khan et al. 2008). Therefore adipose tissue is an excellent source of cells with potential for auricular tissue engineering.

Tissue banks (or biobanks) represent a broad and heterogeneous range of facilities. They may include small collections of tissue with minimal associated data held by a single institution, through to population wide biobanks collecting a variety of tissue and specimen types along with comprehensive medical and epidemiological data (Asslaber & Zatloukal 2007; Riegman et al. 2008). An example of the latter is The UK Biobank that is a multisource-funded initiative that has recruited over 500,000 participants and was opened to access by researchers in 2012 (www.ukbiobank.ac.uk).

In their broadest context, tissue banks collect and store biological materials that may be accompanied with varying amounts of medical, demographic and epidemiological data. They are linked with current (planned) research, but also serve to facilitate future research. They are continuous projects where material and data are collected on a prospective and long term basis. They apply procedures to ensure anonymity, whilst under specific conditions
allow clinically relevant information to be traced back to the donor and accessed by authorised parties. They serve to protect the rights of donors and the interests of researchers by incorporating relevant governance legislation (eg. ethical committee review) and procedures (eg. anonymity and consent) (Yuille et al. 2009). It is this last feature that distinguishes a biobank – a facility that facilitates access to outsiders - from a research collection (Gottweis & Petersen 2008).

The integrated research application system (IRAS) provides a single point of application for all research ethics applications in the UK. It is supported by many of the research-associated charities, organisations and legislative bodies including the Human Tissue Authority and NHS Research & Development. Apart from receiving applications for ethical review, the REC are a valuable source of information and advice to facilitate good practice.

The application system is accessed via their website (https://www.myresearchproject.org.uk) where applicants must register an account prior to submitting an application. Once registered, applicants are required to complete a Project Filter Questionnaire that asks basic questions about the proposal, and based on these answers compiles a project specific application form. This application form is a mandatory part of the final application along with other documents including:
• Application form (mandatory)
• Protocol for management of the tissue bank (mandatory)
• Participant Information Sheet
• Participant Consent Form
• HTA License

The Application Form

Based on the answers given in the Project Filter Questionnaire, an application form specific to the proposed project is generated. This contains general information such as where the project will take place, and those persons responsible for the project. There are also a number of questions that specifically address the issues relating to tissue banking including;

• Tissue
  o What type(s) of tissue are to be collected
  o Where will this tissue be collected
  o What is the intended use of this tissue

• Consent
  o What is the planned process of consent
  o Does the project include vulnerable adults or children unable to consent

• Confidentiality and access to data
  o What level of confidentiality is proposed
  o Who will have access to this data
What safeguards are in place to provide this

If relevant how will relevant findings be notified to patients

- Access to tissue
  - How will external researchers access tissue/data and what processes are in place to facilitate this
  - What information will be released with the tissue
  - Does the bank want to seek generic ethical approval for all subsequent research
  - Is there any potential commercial exploitation of the tissue

The Human Tissue Act

The collection, storage and subsequent use of human tissue is regulated in England, Wales and Northern Ireland by The Human Tissue Act (2004), and in Scotland by The Human Tissue (Scotland) Act 2006. The legislation in Scotland focuses on three principal areas:

1. Organ / tissue donation for the purposes of transplantation, research, teaching and audit

2. Organ / tissue removal and storage as part of post mortem examination.


Although The Human Tissue (Scotland) Act 2006 aims to address the use of tissue for research, it does not directly address the issue of donation of
surplus tissue from live donors for research - the most likely scenario in adipose tissue banking. However, it does refer to the use of human tissue from the deceased for research, and also the use of tissue from live donors for transplant. In each of these occasions it states that the stance of the Scotland Act is to promote equity across the UK, and that their legislation mirrors that of the Human Tissue Act (2004). The HTA requires all tissue banks to have a license from the HTA (except in Scotland). Most NHS and research sites will have a HTA license and the local tissue governance team were contacted prior to the application to get details of this.

There are no formal or legal requirements for tissue banks to obtain ethical approval under the Human Tissue Act or NHS research and governance systems. However any research using the tissue will require individual project based ethical approval. If a RTB seeks ethical approval there is an option to seek generic ethical approval prospectively for any subsequent research using the tissue. Under these circumstances the establishment and/or persons responsible for the RTB assume responsibility for only releasing tissue to third parties whose planned research meets the conditions of the original RTB ethical approval. This has many benefits if the bank plans to supply multiple similar but unrelated projects, and prevents repeated applications to the REC. An example would be to specify that tissue is to be used in research projects within the field of regenerative medicine. This would satisfy the REC that the research was focused, but also allow multiple different and diverse projects to benefit.
Consent

Consent is the process by which a person gives permission for an action to happen. In this context it refers to patients consenting to donate their tissue for research. For this consent to be informed and valid, it must be an autonomous action by a subject that authorises a professional to involve the subject in research. Informed consent is given if:

“a subject with substantial understanding in the absence of control by others intentionally authorizes a professional to perform a procedure.”

(Ruth R. Faden Johns Hopkins University School of Public HealthTom L. Beauchamp Georgetown University 1986).

It is stated by the General Medical Council (GMC) in their publication Good Medical Practice that any doctors who are involved in research:

“must be satisfied that you have consent or other valid authority before you carry out any examination or investigation, provide treatment or involve patients or volunteers in teaching or research.”

This includes the use of patients' organs and tissues.

It might be assumed that consent is a mandatory requirement under all circumstances, however this is not the case. Consent is always required to remove tissue from the deceased for research purposes under the HTA. It is
also required under common law to remove tissue from the living; however this consent may be for a diagnostic or therapeutic intervention and not explicitly for research. Specific consent for use of tissue in research is not required if the tissue is from a live donor, who can’t be identified by the researcher, and the research has been ethically approved. Other significant exceptions under the HTA include:

- Tissue which was collected prior to the introduction of the HTA
- Imported tissue

Despite this, it is regarded as good practice to obtain consent when collecting tissue from living donors wherever possible (Andrews 2005), and that this consent should be separate to the consent gained for the therapeutic or diagnostic intervention.

**Confidentiality**

Confidentiality refers to the promise not to disclose information about the donor that they do not wish to be disclosed. Whilst this is of paramount importance, there are a number of practical and ethical issues that should be considered. For example, if absolute confidentiality is desired this is logistically very difficult if not impossible to achieve. Additionally, any findings associated with the tissue that cannot be linked back to information regarding the sample or to the patient, may limit its utility.
For the purposes of detailing confidentiality, a nomenclature of hierarchy has been established (Bauer et al. 2004):

- **Anonymous**: The source of the tissue was never known.
- **Anonymised**: The identity of the source of the tissue is irreversibly unlinked from the specimen.
- **Identifiable or linked anonymised**: The tissue and donor are indirectly linked (e.g. an ID number), but the identity can only be found by tracing that link.
- **Identified**: The tissue source is known and the identity is directly linked to the sample.

In practical terms, the level of confidentiality should be tailored to the intended applications of the tissue bank and those persons or institutions wishing to utilise it. For research that focuses on specific conditions or diseases then the ability to link that specimen with demographics and medical data is essential and therefore anonymous donation may be undesirable. It has even been argued that under these circumstances, doctors have a moral obligation to ensure that the relevant data is collected to maximize the benefit derived from the tissue (Secko et al. 2009). If confidential information, such as that disclosed in medical records, is to be accessed, then further safeguards will be required to protect the confidentiality of the donor and anyone else associated with that data. However, if the intended use does not require medical and demographic
data, then anonymous or anonymised donation may be preferable. In the case of an adipose tissue bank, such as the one proposed for the work in this thesis, the principle purpose is to provide cells for research into regenerative medicine and tissue engineering. Therefore it is unlikely that any significant amount of demographic or medical data is necessary. Patients undergoing elective cosmetic surgery represent a fairly unique cohort as they are predominantly female, middle aged and free from significant disease and pathology (West et al. 2014).

Whatever standard of confidentiality is proposed and adopted by a tissue bank, it should be explicitly detailed and explained to the donor as part of the consent process. In addition, satisfactory infrastructure should be in place to minimise breaches in confidentiality and also deal with them if and when they occur.

Commercialisation

There is a general sense of anxiety surrounding the commercialisation of human tissue. Many believe that seeking to make profit from human tissue transgresses the principles of altruistic donation on which we rely (Wilkinson 2005). However there are clear benefits, not just financial, that can be gained through commercialisation.

The MRC and GMC state that there should be no financial incentives made to the donors, as this may compromise the process of informed consent.
When there is a financial incentive to participate in research or donate tissue this may affect the voluntary nature of taking part. It is also likely to influence people to do things or accept risks that they would not do were that incentive to be removed. Payment of reasonable costs and expenses that are incurred by the participant are entirely appropriate (MRC 2001).

Both the GMC and the MRC are very clear in stating that human tissue should not be used for financial gain (MRC 2001). Any financial remuneration for supplying tissue to third parties should be limited to recovering administrative costs and doctors should not be involved directly, or indirectly in buying or selling tissues. There are a number of private sector companies operating within the UK offering monetary donations to surgeons able to supply tissue, including adipose tissue. As previously stated, this money should be used to recover costs and associated administration charges and not for personal gain.

Any intellectual property that is gained from research using human tissue can legitimately be exploited for financial gain. Indeed it should be encouraged and facilitated. Commercial sector access to human tissue is fundamental to the discovery and delivery of new medicines and pioneering techniques, which are of immense benefit to the public. It also provides industry with additional incentives to invest in academic research that might otherwise not receive funding (MRC 2001).
There is a general grey area surrounding what amount of intellectual property needs to be added to human tissue to allow it to be a commercially exploitable commodity. Some argue that the labour associated with collecting, processing and storing tissue is in itself enough to allow the tissue to be sold. However, others suggest that significant changes from the starting tissue, such as the generation of immortalised cell lines, are required to satisfy the addition of substantial intellectual property (Lock 2001; Andrews 2001).

It is fundamental to the consent process that any planned or potential commercial activity relating to the tissue is fully explained to the participants. It should also be stated that the participant is not entitled to any share of any profits derived from the tissue.

3.1.1 Summary

Regenerative medicine and tissue engineering is a rapidly expanding area of medical research that shows huge potential in the treatment of many diseases including microtia and acquired ear deformities. Access to ethically sourced human tissue is fundamental to research and the ultimate delivery of successful new treatments. At the outset of this work there was no provision to collect or store adipose tissue, or adipose derived stem cells within the University of Edinburgh or NHS Lothian.
Adipose tissue represents an excellent potential source of stem cells due to the relative ease with which it can be safely harvested and the quantities of tissue available. Studies have also demonstrated the multipotential ability of ASC and the subsets of cells contained within it. Studies examining the potential role of adipose tissue and adipose derived stem cells specific to chondrogenesis and cartilage tissue engineering have demonstrated the chondrogenic potential of ASC, and also identified chondrogenic subsets of cells within the heterogeneous ASC population.

3.1.2 Aims

1. To investigate the attitudes of patients towards the donation of adipose tissue for research.

2. To use this knowledge to design appropriate patient information sheets and consent forms, and to decide the most appropriate model for a tissue bank.

3. To apply for ethical approval to establish a biobank of adipose tissue and adipose derived cells with the appropriate ethical approval for all subsequent research.

4. To establish a working biobank of human adipose tissue and adipose derived cells.
3.2 Patient questionnaire

Adipose tissue is a unique source of stem cells as it is frequently removed in large quantities and discarded as part of routine cosmetic procedures. It is therefore an ideal source, and patients undergoing cosmetic surgery procedures to remove surplus fat make ideal potential donors. In order to gain a better understanding of the perceptions of patients towards donating tissue for research, and also to help in the design of effective and appropriate information sheets and consent forms, a questionnaire was designed by myself to distribute to plastic surgery patients (Figure 3.1). The questionnaire aimed to gain a basic understanding of what patients know about stem cells, stem cell research and general attitudes towards tissue donation. It then focused on the areas highlighted earlier in this chapter including;

- Consent and specifically how much information patients would like to know.
- Confidentiality and if issues relating to this would influence a potential donors decisions.
- The intended use of the tissue including non-medical research and animal research
- Distribution and commercialisation of tissue

Following ethical approval (Formal amendment to SESREC03 10/S1103/45), the questionnaire was distributed to patients attending outpatient appointments at the Spire Hospital, Murrayfield.
**Figure 3.1:** Questionnaire distributed to patients attending plastic surgery outpatients appointments.
Figure 3.1: Questionnaire distributed to patients attending plastic surgery outpatients appointments.
Donating Fat for Medical Research (Version 1.1 – 15.4.11)

14. Would you be happy for your tissue to be shared with researchers other than those collecting the tissue?
   - I would only want my tissue to be used by the research group who collected the tissue.
   - I would be happy for my tissue to be used by other researchers based in the same university/institution.
   - I would be happy for my tissue to be shared with researchers worldwide.

15. The next step in developing new treatments using stem cells would be to begin clinical trials in humans. Would you be happy for your cells to be used as a treatment in other humans? YES/NO

16. Are you on any existing donor register? (Tick all that apply)
   - Yes, I am on the organ donor register
   - Yes, I am on the blood donor register
   - Yes, I am on the bone marrow register
   - No, I am not on any donor registers

If YES, have you donated any tissue before? (Tick all that apply)
   - Organ(s)
   - Blood
   - Bone Marrow

Thank you for your time.

Figure 3.1: Questionnaire distributed to patients attending plastic surgery outpatients appointments.
3.2.1 Patient details

A total of 50 questionnaires were completed with 1 patient declining to participate. 35 (70%) respondents were female, with the mean age being 45 years (range 22-88) which represents a typical cohort of cosmetic surgery patients. 34% of patients were on an existing donor register for blood (20%), bone marrow (14%) and organs (20%), seven of whom (14%) having previously donated blood.

<table>
<thead>
<tr>
<th>Demographic data of patients responding to questionnaire</th>
</tr>
</thead>
</table>
| **Sex** | Female = 35  
| Male = 15 |
| **Age (Years)** | Mean = 45  (Range 22 – 88) |
| **BMI (kg/m²)** | Mean = 25.6  (Range 20.5 – 35.3) |
| **Procedure** | Abdominoplasty = 36  
| Liposuction = 17 |
| Arm / thigh reduction = 5  
| Other = 4 |
| (Some patients had more than 1 procedure) |
| **Registered donor** | Blood = 10  
| Organs = 10 |
| Bone marrow = 7 |

Table 3.1. Demographic data of patients responding to questionnaire. Questionnaires were distributed to patients attending outpatient appointments at Spire Murrayfield Hospital. A total of 50 patients responded with 1 declining to participate.
3.2.2 Stem cell knowledge

Forty one (82%) patients asked had heard of stem cells and were aware of stem cell research. Despite this broad general awareness, only one patient (2%) was aware that adipose tissue was a source of stem cells, compared with bone marrow (28%), umbilical cord (22%), blood (24%), embryos (16%), skin (14%) and muscle (14%) (Figure 3.2). Of those patients that had heard of stem cells, the majority (73%) were aware due to media coverage, with seven patients (17%) being aware through a family members, and four patients (10%) learning about it through work. The one patient who was aware that adipose tissue was a potential source of stem cells knew this due to online research done when a family member required stem cell therapy.
Figure 3.2. Patients understanding of stem cell sources. N=50 patients responded to a questionnaire designed to ascertain their prior understanding of stem cells and stem cells research. This graph demonstrates the percentage of patients who believed stem cells were present in the respective tissues. Embryos = 16%, skin = 14%, muscle = 14%, fat = 2%, umbilical cord = 2%, blood = 24%, bone marrow = 28%.

To further analyse the results of the questionnaire, patients were divided into groups based upon their perceived knowledge of stem cells. Those who were aware of stem cells and had correctly identified 3 or more tissues in which they are present were grouped as having “good” knowledge (n = 18), and those who were not aware of stem cells or only able to name 1 correct tissue were grouped as having “poor” knowledge (n = 26). Differences between groups were analysed using Chi-Square and Mann-Whitney U tests.
No significant differences were noted between the groups based on age (Good; mean = 38.5 years, range 22 – 60. Poor; mean = 41.7 years, range 24 - 88) or sex. Significant differences were observed between the good and poor groups with respect to whether or not they were existing blood/tissue donors. 56% of patients with good knowledge were existing donors compared to 15% of those with poor knowledge (p = 0.028).

3.2.3 Factors influencing patients decisions to donate

When patients were made aware that adipose tissue was a source of stem cells that could be used for research and that these cells were potential targets for novel therapies in regenerative medicine, they all stated that they would be happy in principle for surplus adipose tissue to be used for research. It was then sought to establish what other factors might influence a person’s decision to donate. 96% of patients stated that the level of anonymisation would not influence their decision to donate and they would be happy for researchers to access all of their medical and demographic data and for this to be directly linked with the specimen. However, 4% said they would only donate if the tissue was anonymised and their medical records were not accessed. There were no differences between these 2 groups based on age, sex or previous stem cell knowledge.

Whilst they stated that it might not influence their decision to donate tissue, twenty-one (42%) patients said they would expect to be told as part of the consent process if their tissue was to be used in either cosmetic, commercial
or animal research. 22% of patients had specific concerns relating to the use of their tissue in animal research, however this was often due to a misunderstanding of exactly the type of research being performed. There were no differences in the attitudes towards the use of tissue in animal research based on age (Yes; mean = 42.2 years, range 22-88 versus No; mean = 37.8 years, range 25 – 45), or previous stem cell knowledge. Interestingly all of those patients who would not want their tissue used for animal research were female however this was not statistically significant. 8% of patients would not want their tissue to be used for non-medical research such as the testing of cosmetics. 12% of patients would not want their tissue to be used for, or shared with parties who intended to exploit it for commercial purposes.

All patients said they would be happy for their tissue to be shared with researchers other than those collecting the tissue irrespective of where the other researchers were based. All patients said they would be happy for their cells to be used to treat other humans.

Of those patients that were questioned, 38% expressed a desire to learn more about stem cell research as a result of completing the questionnaire. Previous stem cell knowledge was not a significant factor in determining if people wanted to know more about stem cell research with equal numbers (n=8) of patients from both the good and poor knowledge groups seeking further information. Significantly, all patients said that they would be more
likely to donate tissue in the future as a result of completing the questionnaire and discussing the relevant issues.

### 3.3 Working model for the Edinburgh tissue bank

Based on the results from the patient questionnaire and the anticipated requirements of the tissue bank, it was decided that the most appropriate model was one where tissue was collected anonymised whereby the link between the tissue and the donor is irreversibly broken. Whilst anonymous collection might have been theoretically more favorable, and there appeared to be no concerns from patients about this type of collection, it was felt that achieving this level of confidentiality was unlikely given the close working proximity of the clinical and research teams, and the need to access operating lists. Furthermore, as the primary proposed purpose of the tissue bank is to be a source of cells for regenerative medicine, and specifically within this project for tissue engineering, it was decided that medical data was not necessary for the RTB in its current proposed format. As such, very basic demographic data (Age, sex, BMI and anatomical site of fat removal) was all that was required. This basic demographic data would not compromise the anonymised tissue collection, and it would limit the amount of data accessed should any breaches in confidentiality occur.

Twenty-two percent of patients raised concerns about the potential use of their tissue in animal experiments and stated that it may adversely influence
their decision to donate. Despite this relatively high number, it was felt that the potential need to perform animal based research was critical and so should be part of the ethical review and hence part of the consent process. It was my personal feeling from speaking to patients that much of their concerns relating to animal testing were due to a misunderstanding of the type of animal research that is commonly performed, and that at present this is almost always essential prior to the translation to human use. Furthermore, the use of animals in research is sometimes required as a surrogate for humans due to a lack of access to human tissue, and whilst their single act of donating fat would not alter the overall need for animals in research, better access to human tissue may eventually reduce this burden.

A small number of patients (12%) stated an objection to their tissue being used for or shared with parties for commercial gain. Again, on discussion the main concern raised by patients was the idea of their tissue being ‘sold’ for personal profit. Given the high costs associated with processing tissue, and the potential to recuperate these costs through sharing of tissues with third parties it was decided that the potential for commercialisation should be incorporated into the ethics application, and that this should be explicitly detailed on the PIS and consent forms. Furthermore an explanation of the type of commercialisation would be of benefit to patients to understand what was planned and to allay many of their fears and concerns.
3.3.1 Patient information sheet (PIS)

This is a fundamental part of the consent process and allows the research team to summarise all the salient issues relating to the study. Ideally the research team should explain all the details with the PIS to the patient, and then give them time to consider their decision, however this is not always practically possible. The PIS should be broken up into small paragraphs and should cover a number of essential issues such as:

- An invitation to participate.
- Details of the proposed work and why the patient has been asked.
- Benefits/risks of participation including a statement that participation is voluntary.
- Details of confidentiality arrangements.
- Plans for the donated tissue (processing and disposal).
- Responsible persons, complaints procedures, contact details.
- Details of the ethical review process.

The PIS used for the Edinburgh tissue bank was written based on the above guidelines, the specific nature of the proposed bank and the issues highlighted by the patient questionnaire. A copy of the final PIS accepted by the REC can be seen in Figure 3.3.
Patient Information Sheet
Adipose tissue (fat) bank

We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the project if you wish. Ask us if there is anything that is not clear or if you would like more information.

What is the study about?

We are trying to establish a tissue bank to store human cells for medical research. We are interested in cells that can be removed from adipose tissue (fat). These include fat cells and stem cells which have the potential to grow into a range of different cell types including bone and cartilage. An example of the type of research would be testing the cells in a range of different environments, and with different chemicals to see which ones helped the cells to grow.

Why have I been invited?

You are scheduled to have surgery which involves the removal of fat eg liposuction, tummy tuck, arm lift, thigh lift. At the end of your operation the fat that is removed would normally be discarded. We would like to use this left over fat and remove cells that we are interested in. We would then store the cells for use in further research.

What will happen if I take part?

If you agree to take part you will be asked to sign a consent form to say that you understand what is being asked and that you are happy to be included. As we only want to use the left over tissue, your operation and treatment will be exactly the same whether or not you choose to take part. Therefore there are no risks associated with taking part other than those of the operation itself. You will not personally benefit from taking part, however the information we get from this study might help improve the treatment of people in the future.

Do I have to take part?

No. Taking part is entirely voluntary and whether you take part or not will not affect any other aspect of your care. You will not be paid for taking part. You

Figure 3.3: Patient information sheet for the Edinburgh Tissue Bank.
Version 1.2 (26.10.10)

will have no claim to any developments from the cells, and as they are stored anonymously it would be impossible to trace.

What are the possible benefits of taking part?

None. You will not personally benefit from taking part, however it may help us to improve the treatment of others in the future. As the tissue is stored anonymously you will have no claim to any of the potential developments from the cells.

What are the possible disadvantages and risks of taking part?

None. The fat we hope to use is left-over tissue, and therefore there are no additional risks in taking part other than those of the operation itself.

Will it be kept confidential?

Yes. We will keep a copy of the consent form in your notes, and one in a secure folder at the research site, but this will be the only record of your participation. The leftover tissue will be anonymised and only your age and sex will be recorded with it. We will not access your medical records or remove any data from them.

What happens to the tissue?

The samples will be taken to the University of Edinburgh. We will then extract and store the cells we are interested in. When we have finished any remaining tissue will be disposed of as it would have been if you had not taken part. The cells will then be used to conduct further research. This may be in establishments outside of the UK including commercial organisations, and might include research with animals. Anybody wanting to use the tissue bank will need to apply for permission. This will only be granted when the applications have been reviewed and comply with the regulations of the tissue bank. The tissue will not be used for DNA analysis or cloning.

Who is responsible for the tissue bank?

The tissue will be stored at The University of Edinburgh in the Centre for Regenerative Medicine. Mr Christopher West is the lead researcher and he receives no additional payments for conducting the research.

Figure 3.3: Patient information sheet for the Edinburgh Tissue Bank.
What will happen to the results of the study?

Research using these cells will be shared with the wider scientific community. Studies will be written up and published in relevant scientific journals. The work may also be presented at scientific meetings. It will not be possible to identify any individual from the results.

What if there is a problem?

If you have any concerns about this study you should contact the research team (see below).

Who has reviewed the study?

A favourable ethical opinion has been given by South East Scotland Research Ethics Committee 03 and NHS R&D approval has been obtained.

If you have any further questions about the study please contact Christopher West on:
(0131 2429240) or email: christopher.west@ed.ac.uk

If you wish to make a complaint at any stage please contact NHS Lothian Complaints dept at:
NHS Lothian
Waverley Gate
Edinburgh
EH1 3EG.
Tel: 0131 536 9000
Fax: 0131 536 9164

Figure 3.3: Patient information sheet for the Edinburgh Tissue Bank.
3.3.2 Consent form

This provides a chance to reiterate the key points from the PIS. A member of the research team as well as the participant should sign it. It is advised that the form is filled in triplicate. One copy should be retained by the patient, one copy stored in the patient’s medical notes, and one copy to be stored in a secure place at the research site. In the case of anonymous or anonymised data, this form should not be transported, stored or associated with the tissue. Based on the findings of the patient questionnaire, it was decided that the potential use of tissue in animal research and for commercial purposes should be highlighted again on the consent form. A copy of the final consent form accepted by the REC can be seen in Figure 3.4.
Figure 3.4: Consent form for the Edinburgh Tissue Bank.
3.3.3 Research protocol

A mandatory part of the application is a detailed research protocol that describes the processes and procedures by which the intended project will abide. For proposals that include a specific project, then a detailed description of the methodology is required. However in the case of a tissue bank which seeks generic rather than specific approval this is not the case and the focus is on demonstrating how the proposed tissue bank will ensure the appropriate procedures are followed to according to the boundaries set out in the application form. It is therefore important to demonstrate a practical understanding of how you intend to establish and implement the day to day working and function of the proposal and anticipate and problems. Figure 3.4 provides a schematic overview of the Research Protocol of the Edinburgh Tissue Bank.
Figure 3.5: Flow diagram of the protocol used in the Edinburgh Adipose Bank. Potential donors identified preoperatively and given PIS to read. On day of surgery patient discusses donation with a member of research team and signs consent form if happy to participate. Tissue is collected at the end of surgery and either frozen immediately or processed and stored for future use and/or distribution.
3.3.4 Infrastructure

In addition to the ethical and legal considerations, there are also a number of practical issues that needed to be addressed. The stages of collection, processing, expansion, purification, storage and shipment of adipose tissue and subsets of cells each require specific equipment and infrastructure.

It is the responsibility of the tissue bank to ensure any data collected, or forms containing patient data are stored in a secure place (e.g. locked filing cabinet) at the research site. If the data/tissue has been collected either as anonymous, anonymised or identifiable, then any patient data should be kept separate from the tissue at all times.

The first stage in the process to extract stem and progenitor cells is the isolation of the stromal vascular fraction (SVF) from the adipose tissue (Figure 3.5). There are a number of commercially available automated machines that have been developed to isolate SVF with many of these doing so with the purpose of returning the SVF to the patient for a range of therapeutic purposes (Pérez-Cano et al. 2012). Manual processing of tissue is quick, simple and cost efficient compared to automated alternatives. Full details of the process developed and used in this thesis can be found in Chapter 4. For research purposes the processing of tissue should be performed in a class 2 biosafety cabinet. Initial stages require the disruption (usually by enzymatic digestion) of the adipose tissue. Centrifugation will then generate a pellet containing the stromal vascular fraction (SVF). The
SVF is a heterogeneous mixture of cells including haematopoietic cells, fibroblasts, endothelial cells and stromal stem/progenitor cells. ASC can be selected based on their adhesion to cultureware. Alternatively, specific subpopulations of cells within the SVF can be purified and isolated using methods such as flow cytometry (Corselli, Crisan, et al. 2013).
1. Lipoaspirate freshly harvested from a patient and allowed to settle. 3 distinct phases can be seen with oil on top, above the fat in the middle, with the liquid phase comprising of infiltration fluid and blood at the bottom.

A.) Following washing with PBS and centrifuging, the lipoaspirate separates into 4 phases with oil on top of the adipose tissue. Below which is the liquid phase of PBS, and in the bottom is a pellet containing mostly RBC.

B.) Following enzymatic digestion and centrifugation the lipoaspirate separates into 4 phases. Oil on top of an adipose/connective tissue layer with fluid beneath. The cell pellet in the bottom contains the SVF (white) and RBC (red) which can be seen as two distinct layers.
Cells that are to be stored should be frozen in cryovials in specific freezing media. Cells can be stored for shorter periods of time (weeks) in a -80°C freezer, however for longer periods storage in liquid nitrogen is recommended.

Much of the equipment required is basic and institutions performing in vitro cell culture are likely to be in possession of most of it.

3.3.5 Collaborations / supply / MTA

Distribution of products with collaborators or other third parties should be done by a formal arrangement called a Material Transfer Agreement (MTA). This serves to ensure that the tissue is only used for permissible purposes agreed in the ethics application and information on the form should include contact details, description of the research and general conditions for the transfer and use of the tissue (Hallmans & Vaught 2010). The letter containing advice on applying for access to tissue from the Edinburgh tissue bank can be found in figure 3.7.
Dear Researcher,

Many thanks for your enquiry regarding the adipose and skin tissue bank.

All applications for the use of tissue from the bank are subject to review by Mr Christopher West Clinical Research Fellow at the University of Edinburgh Centre for Regenerative Medicine, and by NHS Lothian Tissue Governance Team.

There are strict conditions concerning the release of tissue from the tissue bank, and you need to be aware of these before applying:

- Tissue is to be used for research within the field of regenerative medicine.
- No tissue should be used in research into termination of pregnancy or reproductive cloning.
- No tissue should be used for the testing of cosmetic products.
- No personal details of the donor will be recorded. Information regarding the sex, age, BMI of the donor are the only details given.
- Research projects in the UK using tissue provided by the bank in accordance with these conditions will be considered to have ethical approval from the committee under the terms of this approval. In England, Wales and Northern Ireland this means that the researcher will not require a licence from the Human Tissue Authority for storage of the tissue in relation to this project.
- Applications from outside the UK should demonstrate they have the relevant local approval for the research.
- Any proposed research using animals requires appropriate local approval.
- A written report detailing the outcomes of any research using tissue from the bank is required on completion of the project.
- Any breeches of these conditions must be reported immediately to Mr Christopher West (c.c.west@doctors.net.uk).

Date:

Figure 3.7. Coverletter and guidance supplied to parties interested in accessing tissue from the tissue bank.
Figure 3.7. Coverletter and guidance supplied to parties interested in accessing tissue from the tissue bank.
3.4 Experiences of the Edinburgh tissue bank

An application to establish an adipose RTB following the model proposed above was submitted through IRAS in September 2011. Following the review, interview and having made the necessary amendments, ethical approval was granted in November 2011.

Since receiving ethical approval, 58 patient samples have been collected. During this period, two patients declined to donate their tissue. On both occasions this was due to an objection to the possibility of their tissue being used in animal research. No concerns or objections were voiced regarding the sharing of tissue with collaborators, potential commercial use of tissue, or the level of confidentiality.

The majority of tissue samples were collected from patients undergoing cosmetic procedures such as liposuction and abdominoplasty in the private sector. A small proportion of samples came from surplus tissue from NHS patients undergoing breast reconstruction procedures. Under the HTA, NHS REC can review and approve applications for RTB using tissue from non-NHS patients such as those having cosmetic procedures in the private sector. Whilst young, healthy donors are the ideal demographic for collecting adipose tissue for the purposes of stem cell isolation (El-ftesi et al. 2009), the unique demographics of these samples may limit their use in the study of some diseases. However, access to human tissue can serve as an ideal
comparison for animal based studies, or as a healthy positive control for disease based research (Esteves et al. 2013).

Tissue collected and stored in the Edinburgh Tissue Bank has been used in numerous collaborations and independent projects both with academic institutions and commercial partners. This has provided a valuable resource for research in many different areas. An overview is found in Figure 3.4, with full details in Appendix 2.

Figure 3.6. UK academic and commercial collaborations with the tissue bank.
3.5 Discussion

Adipose tissue provides an abundant and accessible source of multipotent, autologous mesenchymal stem cells (MSC) for clinical translation. Ease of access, and their ability to be readily expanded in vitro has seen a dramatic rise in the number of clinical trials aiming to harness their regenerative, immunogenic and angiogenic potential. The global recognition of this potential has seen growing use and commercialisation of ADSC in a widespread of clinical settings, from cosmetic breast augmentation, anti-aging and depressed scar therapies, to regenerating and engineering lost tissue after trauma and malignancy. Despite this perceived expansion in our understanding of these cells, they remain poorly understood. This has prompted stringent guidance from the US Food and Drug Administration (FDA) and European Regulation in 2011 for the preparation and processing of ADSC in accordance with current good manufacturing guidelines. Approved stem cell technology must be evaluated with regards to safety, purity, identity, potency and efficacy prior to biologic licensing and clinical use. Therefore access to ethically sourced tissue for research is fundamental to the successful delivery of novel therapies.

The data presented in this chapter from the patient questionnaire demonstrates a significant lack of awareness of the potential use of adipose tissue and ADSC in medical research in a cohort of plastic surgery patients. There is however good potential acceptance for their use following simple provision of information, explanation and opportunity for understanding. This
highlights the significant positive impact engaging with the public and patients can have on the ability to procure tissue for medical research. The analysis of patient knowledge, concerns and expectations surrounding donation of adipose tissue from this study facilitated the design of tailored patient information and consent forms. Gaining information such as this will in turn improve the ability of researchers to engage with public and patients, thus enhancing patient understanding with this highly eligible population of adipose tissue donors. Data from this study suggests that this increased understanding is likely to facilitate access to ethically sourced adipose tissue for future research and therapies. It also demonstrates a general lack of understanding in stem cell knowledge and therefore is applicable other tissues, and stem cell research in general.

As previously discussed, the laws and regulations surrounding gaining ethical and legal access to tissue are often poorly understood, and this may impede access to tissue for researchers. In establishing this tissue bank these regulations were examined to ensure the protection of collaborators, patients and myself. Through developing collaborations between plastic surgeons, private healthcare providers, industry and academic institutions a biobank has been developed that serves to provide cells and tissues to multiple research groups both within the UK, and across the globe. It has also facilitated greater collaboration between clinicians, academics and commercial organisations within the University of Edinburgh and beyond, thus resulting in many new joint projects, grant applications and publications.
4 Identification, isolation and analysis of frequency of pericytes within human adipose tissue.

Parts of this chapter were published in:

**West CC**, Khan NS, Crisan M

*Methods in Molecular Biology* (In press)

Characterisation of Pericyte Phenotype *in vivo* by Immunohistochemistry

NS Khan, **West CC**, Rossi F, Crisan M

*Methods in Molecular Biology* (In press)

Assessment of Pericyte Phenotype by Flow Cytometry

**CC West**, WR Hardy, IR Murray, A James, M Corselli, S Pang, C Black, SE Lobo, K Sukhija, P Liang, V Lagishetty, DC Hay, KL March, E Ting, C Soo, B Péault.

*Stem Cells Research and Therapy*. Revisions made and under 2nd review. October 2015.

Purification of perivascular presumptive mesenchymal stem cells from human adipose tissue: process optimization and cell population metrics across a large cohort of diverse demographics
Corselli C, Crisan M, Murray IR, West CC, Scholes J, Codrea F, Khan N, Péault B


Identification of mesenchymal stem/progenitor cells by flow cytometry.
4.1 Introduction

In a pioneering series of experiments in the 1960s, Friedenstein and colleagues identified a population of cells from rodent bone marrow that were adherent to plastic when grown under standard conditions, formed colonies, and could differentiate into osteoblasts in vitro (Friedenstein et al. 1970). In addition they were capable of generating bone when implanted into ectopic location in vivo (Friedenstein et al. 1966; Friedenstein et al. 1974). Friedenstein termed these cells Colony Forming Unit-Fibroblast (CFU-F), which they were commonly known as until more recently Caplan suggested the term Mesenchymal Stem Cells (MSC) (Caplan 1991). Since this initial description, these cells have been the focus of much attention for their ability to differentiate into multiple mesodermal lineages, to modulate the immune system and to stimulate regeneration through trophic support and secretion of cytokines.

Despite the ultimate desire to translate MSC research to novel clinical therapies, our understanding of these cells is based principally on observations made on in vitro populations and assays, with very little understanding of the in vivo location and identity of these cells. Hence their role in physiological and pathological processes remains relatively unknown.

Since their initial description and derivation from bone marrow, identical cells have been derived from multiple tissues based on their adherence to plastic (da Silva Meirelles 2006). This lead to the hypothesis that these cells may
have a common *in vivo* location and identity. Crisan et al demonstrated that microvascular pericytes in multiple foetal and adult tissues express MSC markers *in vivo*, and when purified to homogeneity and cultured *in vitro* were identical to conventional MSC in terms of morphology, phenotype and function (Crisan et al. 2008). This lead to the conclusion that pericytes represent an *in vivo* ancestor of the MSC observed *in vitro*, a finding that has been validated by other groups (Covas et al. 2008). Subsequently, a second population of anatomically and phenotypically distinct cells with identical function to conventional MSC have been identified that reside in the adventitial layer of larger blood vessels – namely adventitial cells (Corselli et al. 2012). Collectively these 2 populations are referred to by the authors as perivascular stem cells (PSC). Since their description, PSC have been tested in a variety of *in vitro* and *in vivo* assays. The potential of these cells has been shown to replicate the known functions of MSC, and PSC have shown to be of equal function, and in some occasions superior to other stem and progenitor populations (Reviewed in Corselli, Crisan, et al. 2013).

Pericytes have demonstrated chondrogenic potential *in vitro* (Zannettino et al. 2007) and *in vivo* in murine models of meniscal injury (Osawa et al. 2013), rib fracture (Brighton & Hunt 1997) and ectopic transplantation (Farrington-Rock 2004; Diaz-Flores, Gutierrez, Gonzalez, et al. 1991). They have been shown to produce ECM proteins essential for cartilage development (Farrington-Rock 2004). When compared to conventional MSC, pericytes
have demonstrated enhanced chondrogenic potential with increased amounts of GAG production (Hagmann et al. 2014; W. S. Khan et al. 2008).

Despite these promising findings, there are a number of challenges that must be addressed to facilitate the successful translation and wider clinical use of MSC. Standard techniques for deriving and enriching MSC based on adherence to plastic, and the low yield of cells from tissues such as bone marrow have necessitated extended periods of ex-vivo culture prior to transplantation. Further to the significant additional resources and expense required to culture cells in GMP compliant conditions, there are potential risks that in vitro expansion introduces. Infection and immunogenicity are potential consequences from the exposure of cells to animal based culture supplements (Gad 2008). Extended periods of in vitro expansion have been shown to adversely affect the function of cells with a reduction in their chondrogenic, adipogenic and osteogenic potential (James, Zara, Zhang, et al. 2012; Yimu Zhao et al. 2012; Wall et al. 2007). Furthermore, higher passage cells show modified and diminished expression of chemokine receptors and adhesion molecules resulting in diminished response to chemokines and high levels of senescence (Ma 2010). Concerns have also been raised about the development of genetic instability and the potential for malignant transformation in cultured cells. In addition there are significantly more stringent regulatory hurdles that must be addressed with the use of cultured cells compared to those that have been exposed to minimal manipulation (Mahalatchimy et al. 2012).
In an attempt to eliminate many of these issues, some groups have investigated the use of adipose derived stromal vascular fraction (SVF) as a source of MSC / progenitor cells. SVF bypasses the need for in vitro culture and may be delivered at the point of use requiring only basic preparation such as enzymatic digestion, washing and centrifugation. Whilst SVF eliminates the requirement of ex vivo expansion, it is a very heterogenous cell population containing endothelial cells, inflammatory cells, fibroblasts as well as significant amounts of cellular debris. This heterogeneity may limit the regenerative potential of SVF when compared with a more homogenous MSC population as has been demonstrated in models of osteogenesis (James, Zara, Corselli, Askarinam, et al. 2012). Additionally, the use of a heterogeneous population of cells makes demonstrating the specific action of the subpopulations and their mechanism of action practically very difficult to achieve.

It is clear therefore that there is a dichotomy in the current approaches to delivering MSC for clinical use. Methods that use in vitro culture as a means of isolation provide a relatively enriched cell population but incur the practical, financial, ethical and regulatory problems this manifests. Those which use SVF do so at the expense of product identity, purity and function. We have therefore sought to develop methods for the prospective isolation of purified, homogenous populations of MSC based on our understanding of the
exact in vivo location and identity of these cells.

Using high speed, multicolour FACS our group has previously purified cells from a range of adult and foetal tissues (Crisan et al. 2008; Corselli, Crisan, et al. 2013). Here the content of the SVF of human adipose tissue is analysed using flow cytometry to determine the potential yield of PSC and to establish if sufficient numbers of PSC can be purified for clinical application. Thus delivering a pure and defined population of cells without the need for ex vivo culture. Furthermore the patient and procedure based variables that may influence this yield are examined.

4.1.1 Summary

Adipose tissue is potentially an abundant source of pericytes that can be harvested relatively easily from patients. Pericytes have been shown to share features with conventional MSC, and are capable of undergoing chondrogenesis. Subsets of MSC expressing pericyte markers have shown to have enhanced chondrogenic potential when compared to standard MSC (W. S. Khan et al. 2008). This makes them potential candidates for auricular tissue engineering.

By identifying the native location and identity of these cells, they can be isolated to homogeneity without the need for ex vivo culture using FACS.
Given the quantity of adipose tissue frequently removed in cosmetic surgery procedures, this may eliminate the need for ex vivo expansion prior to transplantation for clinical application. This has a number of potential advantages over using cultured cells including

4.1.2 Aims

The specific aims of this experimental chapter are:

1. Using immunohistochemistry to characterise and demonstrate the in vivo identity and location of pericytes within adipose tissue.

2. To develop and optimise protocols for the efficient isolation and purification of pericytes using fluorescence activated cell sorting (FACS) and subsequent culture of pericytes in vitro.

3. In vitro populations of pericytes will be characterised with reference to published data on MSC function and phenotype.

4. The potential numbers of pericytes that can be isolated directly from adipose tissue will be assessed using FACS to determine if sufficient quantities of cells can be purified to eliminate the need for ex vivo expansion prior to clinical use, and in this instance specifically auricular reconstruction.
4.2 Identification of pericytes by immunohistochemistry

A series of immunohistochemical stains were performed to establish the presence of pericytes, their relationship to endothelial cells (EC), and their expression of MSC associated markers. Endothelial markers CD31 (PECAM-1) and vWF were chosen due to the specificity for EC. CD146 (MCAM) is ubiquitously expressed on pericytes, as are markers NG2, αSMA and PDGFRβ (Crisan et al. 2008). CD105 and CD90 are markers associated with MSC and form part of their defining criteria (Dominici et al. 2006).

<table>
<thead>
<tr>
<th>Endothelial cell marker</th>
<th>Pericyte marker</th>
<th>MSC marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31</td>
<td>CD146</td>
<td>CD105</td>
</tr>
<tr>
<td>vWF</td>
<td>NG2</td>
<td>CD90</td>
</tr>
<tr>
<td></td>
<td>αSMA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PDGFRβ</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1: Markers used in the identification and characterization of pericytes in adipose tissue

Immunohistochemistry demonstrated the intimate relationship between endothelial cells and pericytes, with pericyte residing on the abluminal surface of endothelial cells in blood vessels of varying size and calibres (Figure 4.1–3). As adipose tissue contains blood vessels of different sizes,
the selection of markers present on pericytes in all vessels is desirable. In Figure 4.1, CD31 expressing endothelial cells (red) can be seen surrounded by CD146 expressing pericytes (green) in transverse sections of vessels.

Figure 4.2 A - C shows higher magnification of microvessels in longitudinal section demonstrating endothelial cells entirely ensheathed with pericytes. Figure 4.2 D shows a small vessel in transverse section. The microvessels in this figure are of small diameter and consist of only pericytes and endothelial cells that represent the majority of vessels within the microcirculation.

**Figure 4.1:** Immunostaining of microvessels within adipose tissue. Transverse sections of blood vessels using indirect staining of pericytes (CD146 – Red) residing on the abluminal surface of endothelial cells (CD31 – Green). Scale bars = 120µm
Figure 4.2: Immunostaining of microvessels within adipose tissue demonstrating the intimate relationship between pericytes (α-SMA – Green) and endothelial cells (CD31 – Red). A – C shows longitudinal sections of microvessels with endothelial cells entirely ensheathed within a layer of pericytes. D shows a smaller vessel in transverse section. Scale bar = 50µm

Figure 4.3 shows co-expression of CD146 (Green - Pericytes and endothelial cells) with NG2 (Red - Pericytes). Whilst CD146 is ubiquitously expressed on pericytes, it is also present in subsets of endothelial cells as can be seen by the 2 concentric rings of green staining, with the outer layer also staining for
NG2 positive pericytes. For this reason when purifying pericytes from mixed populations of cells containing endothelial cells combinations of markers that are able to distinguish pericytes from endothelial cells would be required.

Figure 4.3: Immunostaining of microvessels in adipose tissue. Co expression of pericyte markers CD146 (Green) and NG2 (Red). Concentric rings of CD146 (green) can be seen demonstrating co-staining of pericytes and endothelial cells with NG2 (red) only staining the outer ring of pericytes. Scale bar = 100 µm.

4.2.1 Co-expression of MSC markers by pericytes in vivo

In the criteria stated for defining MSC, cells are required to express MSC markers CD44, CD73, CD90 and CD105. Although these criteria are used to define in vitro populations of cells, demonstration that pericytes express these markers in vivo would support their role as native MSC, and also to the
hypothesis that these cells could be used without expansion \textit{in vitro}. Using markers confirmed to be pericyte specific in section 4.2, cells also demonstrated co-expression of markers CD105 (Figure 4.5) and CD90 (Figure 4.6).

\textbf{Figure 4.5: Immunostaining of pericytes in adipose tissue.} Demonstration of the native co-expression of MSC marker CD105 (Red) with pericyte marker PDGFR-B (Green). Scale bar = 100µm.
Figure 4.6: Immunostaining of microvessels in adipose tissue. Native co-expression of pericytes (αSMA - Green), with MSC marker (CD90 – Red) in vessels of a variety of sizes and caliber. Scale bars; A = 50μm, B = 100μm, C = 120μm.

4.3 Purification of pericytes by FACS

To further analyse the potential of pericytes, *in vitro* populations of cells were established for further experimentation. To do this, a multi-colour FACS protocol for the purification of viable pericytes from adipose tissue was
developed. For the full protocol on the preparation of SVF see Chapter 2. Following mechanical and enzymatic digestion, cells were washed, filtered and stained with the following antibodies:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorochrome</th>
<th>Intended cell</th>
<th>Off target cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31 or CD144</td>
<td>PE or PERCP-Cy5.5</td>
<td>Endothelial cell</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>CD34</td>
<td>FITC</td>
<td>Adventitial cell</td>
<td>Haematopoietic stem cells</td>
</tr>
<tr>
<td>CD45</td>
<td>APC-Cy7</td>
<td>Haematopoietic</td>
<td>Endothelial cells, B and T lymphocytes</td>
</tr>
<tr>
<td>CD146</td>
<td>APC</td>
<td>Pericyte</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2: Antibody and fluorochrome panel for the purification of pericytes from adipose tissue.

FACS was performed using a BD FACS Aria II following adequate compensation with compensation beads, or single stained cells. Initial gate is forward scatter (FSC) versus side scatter (SSC). FSC is a representation of the density of the events passing through the laser, whilst SSC is a representation of their granularity. By plotting FSC v SSC it is possible to identify distinct populations of cells that can be used as a reference point for subsequent gates. Lymphocytes are small, dense cells that are relatively
homogenous resulting in a small population located in the lower left section of the plot (Figure 4.7 A & B). All viable cells will be above and to the right of this (Figure 4.7 C).

**Figure 4.7. Flow cytometric analysis of SVF from human adipose tissue.** A.) FSC v SSC demonstrating the population of lymphocytes (arrow). B.) Confirmation of the lymphocytes by demonstration of CD45+ phenotype of the sub-population. C.) Selection of the cellular fraction for subsequent analysis.

By plotting the FSC-Height (FSC-H) versus FSC-Area (FSC-A) of cellular gate seen in Figure 4.7C, aggregates of double or multiple cells can be removed to ensure all remaining cells are single (Figure 4.8). In a population of single cells there should be a linear relationship between the magnitude of the signal (FSC-H) and it’s differential (FSC-A) as the size of the cell increases. However, if 2 cells are joined together then the magnitude of the signal remains equal to that of a single cell, however the area increases (Figure 4.8 B).
Table 4.8. Flow cytometric analysis of SVF from human adipose tissue after the selection of cells from initial gates. A.) FSC-A versus FSC-H demonstrating the majority of events following a linear pattern. B.) Diagram showing the linear relationship between single cells but not multiple cells. C.) Gate applied to eliminate multiple cells.

DAPI was used to exclude dead cells, before the selective removal of haematopoietic and endothelial cells by depleting the CD45+ and CD31+ populations respectively (Figure 4.9).

Table 4.9. Flow cytometric analysis of SVF from human adipose tissue. Elimination and selection of subpopulations based on staining. A.) DAPI negative live cells selected. B.) CD45- cells selected to exclude haematopoietic cells. C.) CD144- cells selected to exclude endothelial cells.

Finally, pericytes can be purified by plotting CD146 versus CD34 and selecting the CD146+/CD34- population (Figure 4.10).
Figure 4.10. Final gate for selection of pericytes. Population of live, single, CD45-, CD31-, CD34-, CD146+ cells.

4.4 In vitro culture of pericytes

Immediately following FACS isolation, pericytes were cultured in vitro. It was observed that the initial conditions that the pericytes were grown in had profound effects on their ability to attach and proliferate. A minimum plating density of 20,000 cells per cm$^2$ was required with a density of 40,000 cells per cm$^2$ being optimal. We also observed that pericytes required gelatin coating to facilitate attachment. Cells grown in standard MSC/ASC media (DMEM+20%FCS+1%P/S), did not proliferate upon attachment and no colonies were observed, however cells grown in EGM-2 media did grow after
attachment. Conversely, unsorted ASC would rapidly attach and proliferate at relatively low density, on un-coated plastic in standard media. These observations would suggest that conventional techniques for deriving MSC/ASC may bias against the selection of pericytes.

4.4.1 Cultured pericytes display MSC like morphology and phenotype

Typically, sorted pericytes remained quiescent for up to 7 days following sorting showing no signs of attachment and proliferation. After this period cells attached and spread out and began to form typical fibroblast like colonies, expanding into a standard monolayer of cells. At this point they are indistinguishable from conventional MSC based on morphology alone.

Using ICC it was observed that pericytes grown in standard culture conditions demonstrate positive expression of pericyte markers CD146, PDGFR-β and αSMA, and the MSC marker CD105 (Figure 4.11). In addition they do not express markers associated with other lineages that could either have contaminated the populations during the sorting process, or be derived from pericytes (Figure 4.12). Typical cells that could contaminate pericyte cultures that are present in the SVF are haematopoietic cells (CD45+) and endothelial cells (CD31+).
Figure 4.11: Immunocytochemical staining of pericytes (Passage 3). Positive expression of CD146, PDGFR-β, αSMA and CD105 (Top row) with matched isotype controls (Bottom row).
Figure 4.12: Immunocytochemical staining of pericytes (Passage 3). Negative expression of CD31 (Endothelial), CD34 (adventitial, endothelial, haematopoietic), and OCT4 (Pluripotent cells) (Top row), with matched isotype controls (Bottom row).
The observations made on immunohistochemical analysis of pericyte cultures were then confirmed using flow cytometry (Figure 4.13), using the protocols described in Chapter 2. Flow cytometry confirmed that the cultures were of pericytes (CD146+, PDGFR-β+, CD31-, CD34-, CD45-), and that they were of high purity with rates consistently of greater than 98%. Flow cytometry further confirmed uniformly high expression of MSC associated markers CD44, CD73, CD90 and CD105. Expression of the MSC markers mirrored the expression of pericytes markers and was consistently greater than 98% which is also one of the ISCT defining criteria for MSC (Dominici et al. 2006)
Figure 4.13: Flow cytometric analysis of cultured pericytes (passage 6) demonstrating high purity of pericytes and uniformly high co-expression of MSC markers. Positive expression of pericyte markers (CD146, PDGFR-B), the positive expression of MSC associated markers (CD44, CD73, CD90, CD105) and the negative expression of markers for endothelial cells (CD31, CD34) and haematopoietic cells (CD34, CD45). Red = stained sample, blue = isotype control.

4.4.2 Cultured pericytes are multipotent

Thus far the in vivo location of pericytes within adipose tissue, and surface markers that can uniquely identify them have been demonstrated. In addition, the in vivo co-expression of markers typically used to identify MSC in vitro have been demonstrated. When purified to homogeneity using FACS and cultured in vitro pericytes retain their unique and specific surface marker profile and retain the expression of MSC markers in the absence of any markers associated with other lineages. To robustly demonstrate the association of pericytes with MSC, multilineage differentiation (adipo-, osteo- and chondro-genesis) which is stated as a defining feature of MSC was demonstrated (Dominici et al. 2006).

The ability of MSC to differentiate into mesodermal lineages is well documented and achieved by the supplementation of culture media with combinations of lineage specific growth factors. Growth factors commonly used to induce adipogenesis include dexamethasone, 3-isobutyl-1-methylxanthine (IBMX) and insulin. For osteogenic induction growth factors include dexamethasone, β-glycerophosphate (BGP) and ascorbic acid.
Chondrogenic differentiation can be induced by supplementation of media with ascorbic acid, dexamethasone, insulin and transforming growth factor β-3. Differentiation of pericytes was performed as described in chapter 2.6

4.4.2.1 Osteogenesis

Pericytes were cultured in 12 well plates in SGM and allowed to reach approximately 25% confluence before induction of osteogenesis. When placed in osteogenic media pericytes proliferated rapidly forming a dense monolayer of cells within 1 week (Fig 4.14 A). Pericytes then began to form small nodules of mineralization that demonstrated strong staining. The number and size of nodules increased throughout the duration of osteogenesis (Figure 4.14 B&C). After 4 weeks there was mineralization throughout the wells in both nodules and the cells forming the monolayer (Figure 4.14 D).
Figure 4.14. **Osteogenesis of adipose derived pericytes.** Pericytes (passage 6) grown in 12 well plates in SGM until 25% confluent and then changed to osteogenic media (StemPro® Osteogenesis differentiation kit, Gibco™), staining with Alizarin Red to demonstrate mineralization A.) After 1 week small nodules of mineralization began to form. B.) Staining after 2 weeks showing denser and larger nodule C.) After 3 weeks, multiple nodules demonstrating mineralization with mineralization also developing in the monolayer of cells. D.) Multiple nodules that coalesce with mineralization throughout the monolayer of cells after 4 weeks.

4.4.2.2 Adipogenesis

Pericytes were cultured in 12 well plates in SGM and allowed to reach approximately 40% confluence before induction of adipogenesis. Higher confluency would result in the cell monolayer becoming over confluent and
detaching before adipogenic induction was complete. Small lipid vacuoles began to appear after 5 days and continued to increase in number for the duration of adipogenic induction. After 2 weeks most cells contained lipid vacuoles and the smaller vacuoles began to coalesce into larger vacuoles. Adipogenesis seemed to be enhanced in areas of high cell density.

Figure 4.15. Adipogenesis of adipose derived pericytes. Pericytes (passage 6) grown in 12 well plates in SGM until 40% confluent and then changed to adipogenic media (StemPro® Adipogenesis differentiation kit, Gibco™). A.) Lipid filled vacuoles began to appear after 5 days and were present in approximately 20% of cells at 1 week. B.) 50% of cells containing vacuoles at 2 weeks. C.) Over 80% of cell containing vacuoles at 3 weeks with appearance of larger vacuoles. D.) Adipogenesis occurred earlier and to greater degree in areas of high confluency.
4.4.2.3 Chondrogenesis

Pericytes were cultured in T75 flasks in SGM until sufficient numbers of cells were obtained. 300,000 cells were required for each pellet and chondrogenesis was induced according to the protocols described in Chapter 2.6.

After centrifugation, the cells formed a small pellet in the base of the 15ml centrifuge tube. Pellets in chondrogenic media grew in size over the period of chondrogenic induction and produced ECM that caused them to stick to the sides of the tubes requiring gentle agitation to free them when media was changed. On histological examination of pellets, concentric rings of ECM could be seen on the outer surface of pellets. Control pellets grown in SGM did not grow or produce ECM. Many of the pellets involuted and disintegrated during the course of induction (Figure 4.16).
Figure 4.16 Chondrogenesis of adipose derived pericytes. Pericytes (Passage 6) were formed in to pellets by centrifuging for 3 minutes at 300g (300,000 cells/pellet). Pellets (n=6) were grown in chondrogenic media (StemPro® Chondrogenesis differentiation kit, Gibco™), changed 3 times per week for a total of 4 weeks. Controls (n=6) were grown in SGM. A.) 24 hours after centrifugation, pellets can been seen in the bottom of 15ml tubes. B.) Alcian Blue staining of pellets after 4 weeks of chondrogenic induction showing positive staining of proteoglycans and ECM production. New tissue can be seen as concentric rings on the outer surface of the pellet. C.) Pellets cultured in chondrogenic media (n=6) were significantly larger than those cultured in SGM (n=3) (p = 0.02, Mann-Whitney).
4.5 Prospective isolation of pericytes by FACS

Given the frequency of these cells within adipose tissue, and the large quantities of adipose tissue that can be safely harvested, adipose tissue was analysed to determine the potential yield of pericytes to determine if sufficient numbers could be isolated to eliminate the need for expansion. Adipose tissue from 22 patients was processed using the protocol described in Chapter 4.3. Details of the patients and their samples and the yield of cells is contained in Table 4.3.
<table>
<thead>
<tr>
<th>ID</th>
<th>Age</th>
<th>Sex</th>
<th>BMI</th>
<th>Tissue</th>
<th>Site</th>
<th>SVF (Cells x 10^6)*</th>
<th>Viability (%)</th>
<th>Pericyte (%)</th>
<th>Yield (Cells x 10^6)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP4</td>
<td>43</td>
<td>F</td>
<td>32</td>
<td>Fat</td>
<td>Abdomen</td>
<td>22</td>
<td>85.7</td>
<td>7.0</td>
<td>29</td>
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<tr>
<td>ADP5</td>
<td>42</td>
<td>F</td>
<td>31</td>
<td>Fat</td>
<td>Abdomen</td>
<td>31</td>
<td>81.6</td>
<td>9.5</td>
<td>32</td>
</tr>
<tr>
<td>ADP6</td>
<td>43</td>
<td>F</td>
<td>30</td>
<td>Fat</td>
<td>Abdomen</td>
<td>36</td>
<td>83.9</td>
<td>7.0</td>
<td>32</td>
</tr>
<tr>
<td>ADP7</td>
<td>42</td>
<td>F</td>
<td>29</td>
<td>Fat</td>
<td>Abdomen</td>
<td>33</td>
<td>88.6</td>
<td>8.8</td>
<td>36</td>
</tr>
</tbody>
</table>

Table 4.3: Complete data set of n=2 adipose tissue donors. 

* Total number of nucleated cells in SVF from 100cc of adipose tissue. ** Estimated yield from 100cc of adipose tissue calculated by (SVF x viability x pericyte).
Table 4.4: Summary of the demographic data from Edinburgh donors and samples.

<table>
<thead>
<tr>
<th>Edinburgh Demographic data (n=22)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Female = 20</td>
</tr>
<tr>
<td></td>
<td>Male = 2</td>
</tr>
<tr>
<td><strong>Age (Years)</strong></td>
<td>Mean = 40.3 (Range 29 - 64)</td>
</tr>
<tr>
<td><strong>BMI (Kg/M²)</strong></td>
<td>Mean = 28.1 (Range 22 - 32)</td>
</tr>
<tr>
<td><strong>Tissue type</strong></td>
<td>Whole fat = 12</td>
</tr>
<tr>
<td></td>
<td>Liposuction = 10</td>
</tr>
<tr>
<td><strong>Anatomical location</strong></td>
<td>Abdomen = 18</td>
</tr>
<tr>
<td></td>
<td>Thigh = 3</td>
</tr>
<tr>
<td></td>
<td>Torso = 2</td>
</tr>
<tr>
<td><strong>SVF (Cells x 10⁶)</strong></td>
<td>Mean = 40.7 (Range 8 - 68)</td>
</tr>
<tr>
<td><strong>Viability (%)</strong></td>
<td>Mean = 85.8 (Range 70.1 - 95.7)</td>
</tr>
<tr>
<td><strong>Pericytes (%)</strong></td>
<td>Mean = 11.1 (Range 4.2 - 26.9)</td>
</tr>
<tr>
<td><strong>Yield (Cells x 10⁵)</strong></td>
<td>Mean = 3.85 (Range 0.54 – 8.86)</td>
</tr>
</tbody>
</table>

### 4.5.1 Accumulation of data from Edinburgh and Los Angeles

All adipose tissue samples processed by the 2 Péault Group Laboratories (Edinburgh and Los Angeles) between 2011 and 2014 were examined to
establish the SVF and stem cell content of adipose tissue and to compare this to the recorded patient and processing variables. All samples processed in Edinburgh were done so by a single operator (myself), there was a total of 5 different operators involved in the processing of tissue in LA, however all flow cytometry data was independently reviewed by myself and colleagues in Edinburgh and LA to ensure consistency. In addition to pericytes, the frequency of a second population of MSC like cells – namely adventitial cells – was recorded. Following on from the initial observations that pericytes represent the in-vivo precursor of the MSC (Crisan et al. 2008), a second population of cells within the adventitial layer of blood vessels was identified which had the same properties (Corselli et al. 2012). These cells are distinguishable from pericytes by their unique surface marker profile (Pericytes: CD146+, CD34-, CD45-, CD31- versus Adventitial: CD146-, CD34+, CD45-, CD31-). Collectively these 2 populations are referred to as perivascular stem cells (PSC).

A total of 131 individual samples were analysed (Edinburgh n=22, Los Angeles n = 109) following the protocols described in section 4.3. A summary of the demographic data is found in Table 4.5.
Combined demographic data (n=131)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
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</tr>
<tr>
<td></td>
<td>Male = 18</td>
</tr>
<tr>
<td><strong>Age (Years)</strong></td>
<td>Mean = 41</td>
</tr>
<tr>
<td></td>
<td>(Range 22 - 64)</td>
</tr>
<tr>
<td><strong>BMI (Kg/M^2)</strong></td>
<td>Mean = 26.4</td>
</tr>
<tr>
<td></td>
<td>(Range 19 – 36)</td>
</tr>
<tr>
<td><strong>SVF (Cells x 10^6)</strong></td>
<td>Mean = 34.7</td>
</tr>
<tr>
<td></td>
<td>(Range 4.7 - 120)</td>
</tr>
<tr>
<td><strong>Viability (%)</strong></td>
<td>Mean = 83%</td>
</tr>
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<td></td>
<td>(Range 36 – 99)</td>
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<tr>
<td><strong>Pericytes (%)</strong></td>
<td>Mean = 8%</td>
</tr>
<tr>
<td></td>
<td>(Range 0 - 55)</td>
</tr>
<tr>
<td><strong>Adventitial cells (%)</strong></td>
<td>Mean = 32.6%</td>
</tr>
<tr>
<td></td>
<td>(Range 3 – 72)</td>
</tr>
<tr>
<td><strong>PSC total (%)</strong></td>
<td>Mean = 40.9%</td>
</tr>
<tr>
<td></td>
<td>(Range 6 – 78)</td>
</tr>
<tr>
<td><strong>PSC Yield (Cells x 10^6)</strong></td>
<td>Mean = 11.6</td>
</tr>
<tr>
<td></td>
<td>(Range 1.1 – 47.1)</td>
</tr>
</tbody>
</table>

Table 4.5: Combined donor and sample data from Edinburgh and UCLA.

The mean yield of SVF from 100cc of lipoaspirate was 34.7 x 10^6 nucleated cells (Range = 4.7 - 120). Within this population, the mean viability was 83% (Range = 36 - 99). Haematopietic cells (CD45+) represented a mean of 31.6% of the total viable SVF. Pericytes represented a mean of 8% (Range 0 - 55), and adventitial cells a mean of 32.6% (Range 3 – 72) of the viable SVF. Therefore collectively PSC represented 40.9% (Range 6 – 78) of the viable SVF. There were no significant differences observed in the yield of
SVF or PSC procured per 100cc of adipose tissue between Edinburgh and Los Angeles (Figure 4.15).

**Figure 4.15.** Comparison of SVF yield (A) and PSC Yield (B) as a percentage of live cells per 100mls of lipoaspirate between UCLA and Edinburgh demonstrating no significant differences between centres.

### 4.5.2 Age, sex, BMI and cold storage time do not affect yield of SVF or PSC.

The mean age was 41 years (range: 22 to 64, n=129). There were no differences observed in either the total number of viable SVF cells, or the proportion of PSC as a percentage of live cells with age with linear correlation coefficients (R) of 0.116 and 0.125, respectively (Figure 4.16 a&b). No statistical difference (p > 0.05) was observed in the yield of viable SVF cells or PSC between genders (Figure 4.16c & 4.16d, respectively). Body mass index (BMI) had no significant effect on either the total yield of SVF cells, or the proportion of PSC as a percentage of live cells (R = -0.05 and -0.05, respectively; Figure 4.16e&f).
Figure 4.16. Linear fit of SVF yield & PSC yield \((x10^6)\) per 100mls of lipoaspirate with respect to donor age \((n=124, R = 0.07 \text{ and } 0.09 \text{ respectively})\) (A & B). One way ANOVA analysis of SVF and PSC yield \((x10^6)\) versus donor gender \((n=131, \text{ male } = 19, \text{ female } = 112)\) with green diamonds reflecting the mean yield (center line) and 95% confidence interval (vertical span) for each gender and the grand mean in gray (C & D). Linear fit of SVF yield (E) and proportion of PSC comprising the SVF (F) with respect to donor BMI \((n=97, R = 0.05 \text{ and } 0.01 \text{ respectively})\).

After surgical removal, adipose tissue was stored at 4°C until processed. The majority of samples were processed within 24 hours following the surgery;
however some samples were stored for up to 7 days. A general decline in the yield and viability of SVF cells was observed with increased cold storage time with linear correlation coefficients \( R \) of -0.05 and -0.20, respectively (Figure 4.17 A&B), however this reduction was primarily observed in the CD45\(^+\) haematopoietic cells \( (R = -0.26; \text{Figure 4.17C}) \). The proportion of viable PSC comprising the SVF remained relatively constant for up to 48 hours with no significant effect upon PSC yield per 100ml of lipoaspirate with respect to time by linear regression analysis \( (R = 0.07; \text{Figure 4.17D}) \).

Figure 4.17. Linear fit of various cell isolation parameters from 100cc of adipose tissue with respect to storage time at 4\(^\circ\)C. Total SVF yield (A), cell viability (B), CD45\(^+\) cells (C) and total PSC (D).
4.5.3 Optimisation and process control

Work in this chapter has demonstrated the ability to deliver populations of pericytes that were subsequently expanded *in vitro* for further characterisation, analysis and experimental work. As interest in these cells has developed and their potential for immediate clinical use was explored, it was observed that large numbers of the cells recovered immediately from FACS were of poor quality, and were in the process of dying. This had been previously overlooked in *in vitro* populations as only the healthy cells would adhere and expand. For PSC to be used immediately after FACS, it is imperative to demonstrate that it is possible to recover pure, viable and consistent yields of cells. Our protocols were therefore refined to maximise not only total cell yield, but maximum viable cell yield, purity and consistency leading to the development of an optimized protocol that is described in Chapters 2 and 4. Using this protocol it has been demonstrated not only the recovery of healthy and pure populations of cells, but that results are under statistical control which is fundamental if the immediate use of these cells is to be considered a viable option for clinical therapies (Figure 4.18).
Figure 4.18: Statistical control chart demonstrating that optimization improved the reproducibility of PSC isolation and confirming that both the UCLA and UK isolation processes are under statistical control. A Levey-Jennings chart depicting individual data points for PSC Yield \(x10^6\) obtained from 100mls of lipoaspirate using the UCLA isolation process, before and after optimization, as compared to the UK process \(n=131\). The central green line represents the general mean and is delimited by upper and lower control limits (red lines) based upon a 3-sigma interval.

4.6 Discussion

Adipose tissue is a unique donor source as it is rich in stem and progenitor cells, and can be harvested in large quantities even from individuals of normal BMI with minimal morbidity. By developing an understanding of the exact identity and location of MSC precursors \textit{in vivo}, the ability to purify to homogeneity two distinct populations of multipotent cells – pericytes and adventitial has been demonstrated. Furthermore, the ability to isolate significant numbers of cells from moderate amounts of lipoaspirate has been
demonstrated, which in theory should be sufficient to address many clinical applications.

In the retrospective analysis of 131 adipose tissue donor samples, a theoretical yield of 11.6 million PSC per 100ml of lipoaspirate was demonstrated; however, an average of 9.3 million PSC per 100ml is a more realistic and consistent estimate of PSC based upon process optimisation and data compiled from two different laboratories. Typical cosmetic liposuction procedures frequently exceed 1 litre, and therefore it can be predicted that sufficient cells can be prospectively isolated to satisfy a range of regenerative applications. Table 4.2 summarises in vivo data from our own group and also clinical trials registered at www.clinicaltrials.gov, and estimates the amount of PSC and hence adipose tissue required for a variety of therapeutic uses. Moderate amounts of adipose tissue would be sufficient to provide cells for many of these applications that include tissue engineering and the localised stimulation of regeneration. For those applications where the application is systemic, or when multiple doses are required, such as graft versus host disease, prospective isolation may not be a viable option, and ex vivo culture would still be necessary. Estimates made suggest that for total ear reconstruction 50 million cells would be required. Based on the data provided in this chapter this could be delivered from 400cc of adipose tissue. This is not a significant amount of lipoaspirate and could be removed safely from adults even in those with a BMI within normal limits. However 400cc is less likely to be achievable in children, and therefore the use of PSC without
prior ex vivo expansion is unlikely to yield sufficient cells for use in paediatric
ear reconstruction.

Whilst this data represents the largest published analysis of SVF and
stem/progenitor cell content, the demographics of this cohort reflect the
unique type of patient undergoing cosmetic plastic surgery, and might not
reflect the demographics of people requiring stem cell therapies. Generally
patients undergoing cosmetic surgery are young to middle age women and
free from any significant co-morbidities. Whilst these results suggest that age
is not a factor in PSC yield, the oldest patient in our study was 69. Therefore
further studies are required on patients at the extremes of age. Furthermore,
this study was limited to looking only at the number and viability of cells and
did not examine their function or potency for particular regenerative
purposes. By selecting a purified population of stem/progenitor cells, it is
more likely to increase the efficacy of these cells by eliminating
contaminating cells, however the effects of patient lifestyle, genetic
background, and other variables on the function of the resulting populations
should be examined as both age and disease have been implicated in
reduced function (El-ftesi et al. 2009). This is particularly relevant if an
allogeneic source of MSC is to be proposed and defended as a viable
alternative to autologous cells.
<table>
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<th>Clinical use</th>
<th>Estimated number of cells required</th>
<th>Amount of fat needed</th>
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<tr>
<td>Cartilage for nasal reconstruction</td>
<td>50 million cells / cm</td>
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<tr>
<td>Scaphoid non-union</td>
<td>25 million cells</td>
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<td>Tibial non-union</td>
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<td>Critical limb ischaemia*</td>
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<td>Graft versus host disease*</td>
<td>0.5 – 13 million cells / kg</td>
<td>20cc</td>
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Table 4.6: Predicted number of cells required for therapeutic application and the required amount of adipose tissue to deliver these numbers directly from FACS without expansion. Figures in green are likely to be deliverable based on the required amounts of adipose tissue, numbers in red would not be deliverable and would therefore require periods of ex vivo expansion to generate the required number of cells prior to use.

Note: Numbers of cells required are based upon estimates from our own data apart from those identified by an * which are based on the numbers from trials registered on www.clinicaltrials.gov. 50 million cells would be required for total ear reconstruction with could be delivered from 400cc of fat.
With respect to the function and application of PSC, at present experimental data are limited to in vitro and small animal studies focusing on haematopoiesis support (Corselli, Chin, et al. 2013), bone (James, Zara, Corselli, Askarinam, et al. 2012; James, Zara, Zhang, et al. 2012), muscle (Park et al. 2011), pulmonary (Pierro et al. 2012), peripheral nerve (Lavasani et al. 2014) and cardiovascular regeneration (Campagnolo et al. 2010; Chen et al. 2012; Katare & Madeddu 2013; Dar et al. 2012; He et al. 2010). In these models, the function of PSC has been shown to be equivalent or superior to conventional MSC or other progenitor populations. A broader assessment of function/potency in a range of assays, and large animal and clinical trials, is required before PSC can be deemed to be equivalent or perhaps superior to conventional MSC in multiple indications. Evidence from other studies demonstrates that pericytes are able to undergo chondrogenesis and produce ECM proteins required to generate mature cartilage (Farrington-Rock 2004). Furthermore, highly chondrogenic subsets of MSC were identified and expressed pericyte markers (W. S. Khan et al. 2008). This data supports the potential application of adipose derived pericytes for auricular tissue engineering.

Technically, the process of purification of PSC in this work relies on sophisticated and costly FACS flow cytometers, a device that is not common in clinical settings; and there are significant costs associated with the acquisition and maintenance of these instruments. Additionally, clinical-grade cell sorters have not been approved by regulatory bodies that govern
clinical procedures although many companies are now actively involved in the development of instruments that address the regulatory requirements necessary to utilise these devices in the clinic.
5 High-throughput microarray screening of a polymer library to identify substrates that support the attachment, proliferation and differentiation of human adipose derived pericytes.

5.1 Introduction

As discussed in chapter 1, there are many different types of biomaterials used in regenerative medicine, each with potential advantages and disadvantages. A major hurdle in the identification of novel materials has been the slow and laborious traditional methods of screening and testing. This has led to the development of high-throughput strategies that allow rapid and automated screening of multiple materials simultaneously and in parallel.

These techniques were pioneered by 2 main research groups, namely the Langer Group (Massachusetts Institute of Technology) and the Bradley Group (University of Edinburgh). Both groups generated extensive libraries of polymers that were printed onto standard microscope slides. Langer originally developed a polymer microarray of 576 polymers made from 25 different acrylate monomers. Using slides coated with poly (HEMA) to prevent non-specific cell binding they incubated hESC for 6 days on the microarrays in the presence of retinoic acid – a known differentiation factor for ectodermal lineages. Through this they were rapidly able to identify
substrates that demonstrated enhanced cytokeratin 7 (epithelial marker) expression (D. G. Anderson et al. 2004). Whilst this study demonstrated the potential of microarrays for cell selection, it did not evaluate the long term or large-scale potential of these substrates.

In contrast, the Bradley Group developed and validated two distinct approaches in the production of microarrays. The first technique uses contact printing of preformed polymers onto the surface of agarose coated slides (Figure 5.1). The agarose coating prevents non-specific adhesion of cells to the slide, and is optimal for this application as it can be easily UV sterilised, and does not dissolve in most organic solvents which would cause leaching into the other polymers during fabrication (Tourniaire et al. 2006). The group invested significant effort in ensuring all stages of the process were fully optimised and characterised to facilitate rapid and efficient identification of substrates followed by immediate scale-up. Variables including the type of solvent, type of substrate, inking time, stamping time, number of stamps per spot and washing conditions were all investigated and shown to have significant effects on the shape, uniformity and reproducibility of the printed spots. Using this approach the group examined multiple different cell types and lines including ESC, iPSC, ADSC and endothelial progenitor cells resulting in the development of in vivo scaffolds for tissue engineering (F. Khan et al. 2010), coatings for biomedical implants (Pernagallo et al. 2012), and pre-clinical tools for drug identification and screening (Hay et al. 2011). Significantly, this approach confirmed the ability to rapidly scale up the
identified polymers and demonstrate large scale and longterm functional benefits.

Figure: 5.1. Microarray printing of polymer libraries on to agarose coated microscope slide as developed by the Bradley Group. A Q-arrayer\textsuperscript{mini} (Genetix, UK) machine, using aQu solid tipped pins (K2785, Genetix, UK) inked from a 384 well plate containing 1% (w/v) polymer solutions in N-methylpyrrolidone (NMP) was used. The central image shows the pins inking from a 384 well plate before transferring to the microscope slide.

The second approach was to develop inkjet fabrication of microarrays in which the polymerisation occurred \textit{in situ} on the microscope slide, as opposed to the printing of preformed polymers as previously described. Monomers, crosslinkers, and initiators can all be printed onto the slide. Traditionally this was achieved using water compatible polymers, but with the use of organic solvents the repertoire of monomer and reagents that can be printed has expanded creating new polymers. The main advantage of this system was that the non-contact approach allowed a greater degree of control over the size and morphology of the printed dots. Using inkjet
fabrication, Hansen et al developed a high density microarray in which over 7000 polymers were printed on a single microscope slide (standard microarrays developed by the Bradley group contain approximately 380 polymers). This was used to identify synthetic substrates for the stable long term culture of hESC (Hansen et al. 2014). In addition to the printing of dots on microarrays, Hansen et al demonstrated the ability to print combinations of 2 or more monomers in gradients. In this experiment 84 polymer gradients were created and screened with 2 different cell lines. The gradients allowed for optimal polymer compositions to be identified from a theoretically infinite number of combinations (Hansen et al. 2012).

To allow rapid analysis of the polymer microarrays, high-resolution high content (HCS) systems have been developed that allow detailed imaging of structures down to 0.2µm. The systems are based on a standard fluorescence microscope attached to a motorised stage. This allows the microarrays to be automatically scanned and multiple images of each spot can be captured in different channels. When used in conjunction with automated image analysis software (such as the Pathfinder™ software package used in this chapter) multiple parameters including cell number, shape, size and fluorescence levels can be ascertained from thousands of images allowing accurate assessment of multiple assays (Figure 5.2).
Figure 5.2. Workflow of the Bradley Group’s high-throughput screening and analysis.

Polymer microarrays printed onto the surface of microscope slides in a variety of methods. Rapid automated screening and analysis to allow early selection of ‘hit’ polymers for scale up and further biomechanical, in vitro and in vivo assessment.

Biomaterials have been used in ear reconstruction in a variety of applications. The first work describing the routine use of a synthetic framework to act as a permanent implant was by Cronin in 1966 (Cronin 1966). Cronin used a soft silastic framework, however he acknowledged the principle problem of extrusion associated with this type of implant due to a lack of biocompatibility, subsequently abandoning the technique in favour of autologous methods.

The most common permanent synthetic framework in current use is Medpor® which is a porous polyethylene scaffold. In the largest published series comparing autologous and Medpor® based reconstructions, 1864 patients were studied and significantly higher extrusion rates were seen in the Medpor® group (13.5%) versus the autologous group (1%). There was
also greater overall satisfaction in the autologous group versus the Medpor® group (98% versus 84% respectively) (Yanyong Zhao et al. 2009).

The commonest group of synthetic polymers used for auricular tissue engineering are aliphatic polyesters such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and poly(caprolactone) (PCL), which are biodegradable through hydrolysis of the ester linkages. These groups have been shown to be biocompatible and support the growth and differentiation of both chondrocytes and stem cell populations (Cao et al. 1997). Problems arising with these polymers come from acidic degradation products that can cause resorption of the engineering structure. Immunogenicity of the degradation products (Nayyer et al. 2012), and insufficient biomechanical strength to maintain the shape of the engineered structure long term in vivo (Shieh et al. 2004) (Cao et al. 1997). Blending of different polymers allows for alterations in their degradation rates and has demonstrated improved biomechanical function in longer terms studies of 20 weeks in vivo implantation (Pomerantseva et al. 2016).

5.1.1 Aims

The aim of this section is to identify potential synthetic polymers that can be used as scaffolds in auricular tissue engineering. In Chapter 4, the ability of adipose derived pericytes to function as multipotent mesenchymal stem cells was demonstrated. By identifying the unique phenotype of these cells they
could be isolated using FACS in sufficient numbers for many clinical applications. However, the numbers of cells required for auricular reconstruction (approx. 50 million) would require an estimated 400cc of lipoaspirate if both PSC populations are used (pericytes and adventitial cells), and 1600cc of lipoaspirate if only pericytes are to be used, which is not feasible in children. Therefore, in addition to demonstrating the ability to bind adipose derived pericytes, the ability of the polymers to support both the long term, stable proliferation of cells and their subsequent differentiation is also necessary.

Polymer microarrays will be used as a high throughput method to screen a library of polyacrylates/acrylamides synthesized from acrylate, acrylamide and vinyl monomers as candidates with high binding affinity for human adipose derived pericytes. In addition, the ability of these substrates to support the stable proliferation of PSC whilst maintaining their phenotype in vitro will assessed by performing IHC and flow cytometry on cells grown on glass coverslips coated with the specific polymers. Furthermore, polymers that are capable of supporting the subsequent differentiation of cells in vitro that may be able to act as substrates for the delivery of these cells in vivo will be identified using quantitative assessment of functional staining for adipogenesis, osteogenesis, and chondrogenesis.

Therefore the specific aims of this chapter are:
1. Use a high throughput approach to screen a library of polyurethanes and polyacrylates to identify substrates with high binding affinity for human adipose derived pericytes

2. Assess the ability of selected polymers to support the stable growth of pericytes in extended periods of *in vitro* culture.

3. Quantitatively analyse the ability of substrates to support and promote the differentiation of pericytes to osteoblasts, adipocytes and chondrocytes.
5.2 Screening of microarrays

Microarrays were created from the polymer library as described in Chapter 2. Cells were seen to adhere to polymer spots (Figure 5.3C), and also consistently adhere to the replicated spots of individual polymers (Figure 5.3A). Figure 5.3 B shows a polymer with no cells attached. A general observation was that the majority of polymers had no, or very few cells attached, however those polymers that did bind cells did so in large numbers. This was reflected in the mean, median and mode number of cells per polymer spot being 3, 2 and 0 respectively, however the range was 0 – 563. Figure 5.3D demonstrates nuclear staining using DAPI which was used to perform automated cell counts. Figure 5.3E demonstrates the retained expression of CD146 by pericytes adhered to the polymer.
Figure 5.3. Polymer and percyte binding after 24 hours. Polymer microarrays of 382 distinct polymers printed onto a single agarose coated glass slide seeded with 500,000 adipose derived pericytes (Passage 3). A.) Pericytes binding to two replicate polymer spots (Brightfield). B.) Polymer demonstrating no cell attachment (Brightfield). C.) Higher magnification of cells bound to polymer spot (Brightfield). D.) Nuclear staining of cells bound to polymer seen through the DAPI channel. E.) ICC staining of cells with CD146 (Abcam Inc) demonstrating retained expression whilst bound to polymer. Scale bar 300µm.

Statistically significant differences were observed between the mean values of all the groups observed (p<0.0001), demonstrating significant differences in the overall binding ability between polymers. Analysis of each individual polymer versus control (no polymer), demonstrated specific polymers with a statistically significantly greater ability to bind pericytes. The results of the
first analysis can be seen in Table 5.1 (NS = Not significant, * = p<0.05, ** = p<0.01, *** = p<0.001).

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**Table 5.1**: Results of the first polymer screen after pericytes had been incubated on the polymer coated glass microscope slide for 24 hours. Total number of cells bound to polymer spots was compared to control areas of no polymer coating. Polymers displaying significantly greater binding affinity (compared to control) highlighted in yellow using Dunnett’s Multiple Comparisons Test. (NS = Not significant, * = p<0.05, ** = p<0.01, *** = p<0.001).
5.2.1 Development of ‘hit’ array

Analysis of the results from the microarray data identified 5 polymers that showed significantly higher binding affinity when compared to control on all 4 screens (Table 5.2).

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Screen</th>
<th>1</th>
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Table 5.2: A summary of the ‘hit’ polymers demonstrating 5 polymers with significantly enhanced binding efficacy compared to control in each of the 4 screens performed using different donor cells (* = p<0.05, ** = p<0.01, *** = p<0.001).

Based on these results from the initial screens, a “hit” array was developed using the same microarray printing techniques described in Chapter 2. The 5 polymers identified in Chapter 5.2.1 were cross-referenced with a library of over 2000 other polymers to identify similar, yet distinct polymers to include in this array (Table 5.3). The “hit” array thus contained 19 polymers each printed in octuplicate with 5 control areas of no polymer.
<table>
<thead>
<tr>
<th>Name</th>
<th>Polymer Structure</th>
<th>Ratio (%)</th>
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</thead>
<tbody>
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</tr>
<tr>
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<td>VP-4</td>
</tr>
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<td>VP-4</td>
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<td>HEMA</td>
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</tr>
<tr>
<td>PA338</td>
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</tbody>
</table>

**Table 5.3:** Generation of a ‘hit’ array. The 5 candidate polymers identified in the initial screen (yellow) were cross-referenced with the extended polymer library to identify similar polymers for further analysis and evaluation.

‘Hit’ polymer screens were performed with 2 separate patient samples (n=2), which were different to the samples used in the initial screen, and each array was performed in duplicate. Slides were fixed and analysed as described in Chapter 2. Analysis of the all the groups demonstrated significant differences (p<0.001) between the groups. As the hit array had been developed from polymers known to have a high binding affinity for pericytes, it was decided to
use the mean value of all the groups tested instead of no polymer as the standard control when analysing specific polymers. Using this method 5 polymers demonstrated significantly greater binding ability compared to the mean of all groups; PA133 (P<0.05), PA136 (P<0.05), PA210 (P<0.05), PA337 (P<0.05), PA338 (P<0.01).

![Analysis of 'hit' polymers](image)

**Figure 5.4:** Analysis of hit polymers showing the total number of cells bound to each polymer after 24 hours incubation. Results represented as box and whisker plots showing mean, range and upper and lower quartiles, with results grouped according to the family of polymers they are from. Mean of all polymers is seen in pink, those polymers demonstrating significantly greater binding than the mean are identified with an asterisk (* = p<0.05, ** = p<0.01).
5.2.2 Intra sample correlation

To determine the reproducibility of the individual polymers ability to bind pericytes, the screening process was repeated with the same patient cell sample on 2 separate occasions. The cells for each experiment were from different stocks and at different passage (Passage 3 and passage 8) in an effort to maximise the heterogeneity between the 2 samples and minimise the chance that the cells were derived from an identical population or clone. Results were calculated as the mean number of cells bound to each replicated polymer spot (n=8). These data were plotted to demonstrate those polymers that displayed the best intra sample correlation. Data was analysed using nonparametric Spearman correlation, and 2 tailed t-test. Results of this experiment demonstrate that there was excellent reproducibility when the same sample was screened at different passages (r = 0.82, 95%CI = 0.69 – 0.9, p<0.0001). Polymers identified from the hit array are highlighted in Figure 5.5 demonstrating both high binding and excellent intra-sample correlation.
Figure 5.5: Assessment of intra-sample correlation represented by comparing the numbers of cells bound to each polymer spot when the screen was repeated with cells from the same donor after 24 hours of incubation. Screen 1 = passage 3 cells, screen 2 = passage 8 cells. Spearman correlation demonstrated strong correlation ($r=0.82$, 95%CI = 0.69 – 0.9, $p<0.0001$) and hence strong reproducibility of the polymers tested. Polymers identified from the hit array are highlighted in the upper right quadrant demonstrating both high binding and excellent intra-sample reproducibility. Data plots grouped according to family of polymers (Blue circle = PA132 – 137, orange diamond = PA186 – 191, green triangle = PA210 – 227, red square = PA336 – 338).

5.2.3 Inter sample correlation

To determine the reproducibility of the individual polymers ability to bind pericytes from different donors, the screening process was performed with
pericytes from 2 separate donors (n=2) and repeated. This information was important to ensure the candidate polymers we selected would bind pericytes from multiple, and not specific donors. Results were calculated as the mean number of cells bound to each replicated polymer spot (n=8). These data were plotted to demonstrate those polymers that displayed the best inter sample correlation. Data was analysed using nonparametric Spearman correlation, and 2 tailed t-test. Results of this experiment demonstrated good reproducibility when the experiment was repeated with different donor samples (r=0.51, 95%CI = 0.12 – 0.76, p = 0.01). Polymers identified from the hit array are highlighted in figure 5.6 demonstrating both high binding and good inter sample correlation.
Figure 5.6: Assessment of inter-sample variation represented by comparing the numbers of cells bound to each polymer spot when the screen was repeated with cells from different donors. Spearman correlation demonstrated good correlation (r=0.51, 95% CI = 0.12 – 0.76, p = 0.01) and hence good reproducibility of the polymers tested. Polymers identified from the hit array are highlighted in the upper right quadrant demonstrating both high binding and good inter-sample reproducibility. Samples were analysed after 24 hours of incubation. Data plots grouped according to family of polymers (Blue circle = PA132 – 137, orange diamond = PA186 – 191, green triangle = PA210 – 227, red square = PA336 – 338).

5.3 *In vitro* effects of polymers on PSC

The 5 candidate polymers were dissolved in solvent (PA133, 136, 337, 338 in THF, and PA210 in NMP) to make a final solution of 2% (w/v). Individual polymers were then spin coated onto the surface of standard glass coverslips using a Spincoater Model P6700 Series (Specialty Coating Systems, Inc).
Slides were dried overnight at 40°C in a vacuum oven (Full details described in Chapter 2). All subsequent experiments were performed with the glass coverslips placed in the bottom of a low adherence 12 well plate (Corning Supplies) unless otherwise specified. A glass coverslip with no polymer coating used as control. Low adherence plates were used to prevent the potential binding of cells to the plate instead of the polymer coated glass coverslip.

5.3.1 Morphology

5000 pericytes in 2ml of SGM were placed onto the surface of polymer coated glass coverslips and differences in morphology were observed. In the control wells with uncoated glass cover slips, pericytes formed an evenly spaced monolayer of cells with typical fibroblast like appearance (Figure 5.7 F). A similar morphology was seen in the cells plated on PA210 coated glass coverslips (Figure 5.7 C). Cells grown on coverslips coated with PA337 and PA338 initially formed a monolayer but subsequently continued to rapidly proliferate and continued to grow past confluency. The resulting cultures demonstrated densely packed layers of overlying cells that apparently lacked the normal cell to cell inhibition (Figure 5.7 D & E). Cells grown on coverslips coated with PA133 developed thin spindle like projections that communicated with other cells, at higher confluency these formed into ridges of cells (Fig 5.7 A). Cells grown on PA136 had similar morphological characteristics to those plated on PA133 (Fig 5.7 B), however in addition they also formed
densely packed spheroidal colonies of cells termed ‘perispheres’ (Fig 5.7 B - yellow box).

Figure 5.7: Morphology of pericytes grown on polymer coated glass cover slips. A.) Cells placed on polymer PA133 with spindle shapes at low density. B.) Cells placed on PA136 showing formation of ‘perispheres’ (Yellow box). C.) Cells placed on polymer PA210 displaying normal morphology. D) & E) Cells placed on PA337 and PA338 respectively rapidly grew to overconfluency with lack of cell to cell inhibition. F.) No polymer (control). Scale bar = 200µm.
Perispheres are a feature that have been previously observed in pericytes grown in standard conditions, however they only appeared after cells had been cultured for long periods *in vitro*, and were left to grow beyond confluence. The appearance of perispheres in cells plated on PA136 occurred immediately and at low density, which has not been previously observed. Other than their early appearance at low density, the perispheres seen on the PA136 coated glass coverslips were indistinguishable from those previously seen. They range in size from 100 - 400µm (Figure 5.8 A&B), are able to detach from the base of the well and become freely mobile and merge with other spheres (Figure 5.8 C), and can repopulate a monolayer of cells when transferred in to an empty culture vessel (Figure 5.8 D).
Figure 5.8: Perispheres formed from cultures of pericytes grown on glass cover slips coated with polymer PA136. A & B Formation of perispheres. C. 2 mobile perispheres merging together. D. A perisphere transferred to an empty uncoated well and regenerating a monolayer of cells (x20).

PA136 coated coverslips with perispheres were placed in 2ml of adipogenic medium (StemPro® Adipogenesis differentiation kit, Gibco™) and media was changed every 48 hours for 1 week. It was observed that the cells migrating out from the periphery of the perisphere were highly adipogenic, however the cells in the centre did not differentiate and appeared to continue to repopulate the sphere (Fig 5.9).
Fig 5.9: Perisphere placed in adipogenic medium for 1 week showing cells emerging from the periphery of the sphere rapidly differentiating into adipocytes (x40). Scale bar = 100µm

To further assess the characteristics of the perispheres, ICC was performed to determine the phenotype of cells in the centre of the perisphere and those emerging from the periphery. ICC was performed using the protocol described in Chapter 2. Perispheres were stained for pericyte markers and also markers associated with pluripotency (Oct-4). Cells at the periphery of the sphere, and those growing out from it demonstrated the normal pericyte phenotype with positive expression of pericyte markers. Interestingly, the cells in the centre of the sphere demonstrated much weaker expression of pericyte markers (Fig 5.10 A & B). When stained with markers associated with pluripotency, cells in the centre of the sphere showed positive
expression of Oct-4, however this expression was absent in cells at the periphery and those growing out from the perispheres (Fig 5.10 C).

![Figure 5.10: Staining of perispheres demonstrating week expression of pericyte markers on cells in the centre of the sphere with strong expression in cells emerging from the periphery (A & B). Oct-4, a marker associated with pluripotency that is not normally expressed by pericytes was also expressed in cells found in the centre of the spheres but absent in those emerging from the periphery (C) (x40).](image)

To further analyse the perispheres, FACS was performed on cells from the perispheres (nodules) and those from the monolayer of cells outside of the nodules. FACS analysis supported the ICC data and demonstrated 89% of
cells in the centre of nodules showing positive Oct-4 expression, but no expression seen in the cells from outside the nodule (Figure 5.11).

To further examine and confirm these observations, the mRNA expression of pluripotency genes Oct4 and Nanog was assessed in cells from perispheres (nodules) compared to the total population. Higher levels of both genes were detected in cells from nodules when compared to cells from the monolayer. Relative expression of Oct-4 was 447 versus 17, and Nanog was 249 versus 14 in cells from nodules and monolayer respectively (Figure 5.11).
Figure 5.11: A. FACS analysis of cells contained within nodules (perispheres) demonstrating 89% of cells with positive expression of Oct4. B. Analysis of cells outside of nodules demonstrating 0% of cells with positive Oct4 expression. C. RT/qPCR of total cell population (purple) versus cells from nodules (crimson) demonstrating higher mRNA expression of Oct4 and Nanog in cells from within nodules. (Data and figures provided by M. Crisan)

5.3.2 Long term culture

The ability of the polymer-coated coverslips to maintain stable pericyte phenotype through extended periods of in vitro culture was assessed using flow cytometry to examine the proportion of cells expressing pericyte specific
and MSC associated surface markers. Pericytes from different donors (n=2) were seeded onto the surface of polymer coated glass coverslips and cultured in SGM at 37°C until the cells became confluent. At this point cells were removed from the polymer coated coverslips with 0.25% trypsin. 20% of recovered cells were replated on new polymer coated coverslips, the remaining 80% were analysed at passage 3, 6 and 9 for their surface marker phenotype by flow cytometry using the protocols described in Chapter 2. Data is represented as the average value from all donors.

Expression of pericytes markers CD146 and PDGFR-β was retained at high levels (>95%) on polymers PA210, 337, 338 and control. A gradual decrease in CD146 positive cells was seen on polymer 133 (92% at passage 9), with a marked decrease seen on polymer 136 (76% at passage 9) (Figure 5.12). This decrease was seen in association with an increase in the formation of perispheres (see previous section).

Figure 5.12. Expression of pericyte markers by cells cultured on polymer coated cover slips.
A. Expression of CD146. B. Expression of PDGFR-β.
Expression of MSC marker CD105 followed a similar pattern to pericyte markers with high levels of expression (>95%) on polymers PA210, 337, 338 and control. A slight decrease observed on polymer PA133 (91% at passage 9) and marked reduction on polymer PA136 (74% at passage 9) (Figure 5.13).

![Expression of CD105 by cultured pericytes](image)

**Figure 5.13.** Expression of CD105 by cells cultured on polymer coated glass coverslips.

Expression of other markers (CD31, CD34, and CD45) was negative throughout all passages on all polymers and control (data not shown).

### 5.3.3 Differentiation

The ability of the individual polymers to support, and potentially enhance the differentiation of pericytes into mesodermal lineages *in vitro* was assessed. Polymer coated glass cover slips were created as detailed in Chapter 2, prior to subsequent experiments. Passage 4 and 5 pericytes from *n*=3 different
donors were seeded on to the polymer-coated glass coverslips at an initial density of 10,000 cells per well for osteogenesis, and 20,000 cells per well for adipo and chondrogenesis in SGM and allowed to attach for 48 hours. Slides were then washed 3 times in PBS to remove dead or non-adherent cells and the media changed to either osteogenic or adipogenic media (StemPro® Adipogenesis / Osteogenesis / Chondrogenesis differentiation kit, Gibco™). Thereafter, media was changed three times each week for a total of 3 weeks for adipo and osteogenesis and 4 weeks for chondrogenesis. At the end of this period, cells were fixed and stained as detailed in Chapter 2.

5.3.3.1 Osteogenesis

Osteogenesis was quantified using an alizarin red elution assay as described in Chapter 2 (Gregory et al. 2004). Briefly, slides were fixed and stained with alizarin red to demonstrate the presence of mineralization as seen by red staining. Alizarin red was then eluted using acetic acid, neutralized with ammonium hydroxide and then quantified using colourimetric detection at 405nm.

Osteogenic differentiation was observed in all wells tested however there were clear variations between patient samples where there was a general trend for ADP31 to show the most evidence of mineralization and ADP37 to show the least (Figure 5.14 A).
General trends were observed in the ability of the polymers to support osteogenesis with PA133, PA337 and PA338 performing better than control, and PA136, PA210 worse than control. There were no significant differences observed between the means of the groups when analysed using the Kruskall-Wallis test. Furthermore, no significant differences were observed when the individual groups were compared with each other using Dunn’s multiple comparisons test.

![Image of osteogenesis on polymer coated glass coverslips](image)

**Figure 5.14. Osteogenesis on polymer coated glass coverslips.** A.) Appearance of cells following 3 weeks of osteogenic differentiation on polymer coated glass coverslips (Stained with Alizarin Red). B.) Elution of staining prior to assessment. C.) Quantitative assessment of osteogenesis using colourimetric assessment of mineralization using Alizarin Red staining. No significant differences were noted between polymers and control.
5.3.3.2 Adipogenesis

Adipogenesis was quantified using an Oil Red O elution assay (Chemicon® International – ECM950). Following 3 weeks of differentiation in Adipogenic growth media cells were fixed and stained with Oil Red O to demonstrate the presence of intracellular lipids. Cells were stained following the protocol described in chapter 2. Following this, 0.5ml of Dye Extraction Solution was added to each well and transferred to an orbital shaker for 15 minutes. 150µl of the solution was then transferred in triplicate to the wells of a 96 well plate and quantified using colourimetric detection at 520nm.

Clear differences in the degree of differentiation were observed between the cells grown on different polymer coatings. This was confirmed on quantitative assessment of adipogenesis. ANOVA (Kruskall-Wallis test) demonstrated significant differences in the means of the groups analysed (P = 0.01), and when Dunn’s multiple comparisons test was applied to compare all groups, significant differences was observed between PA133 (worst performing) and PA338 (best performing) (P < 0.01).

PA337 and PA338 both showed greater amounts of lipid production compared to control (no polymer) however the differences were not significant.
Figure 5.15. Adipogenesis on polymer coated glass coverslips. A.) Appearance of cells before staining following 3 weeks of adipogenic differentiation on polymer coated glass coverslips. Intracellular lipid droplets can be seen. Scale bar 200µm B.) Quantitative assessment of adipogenesis using colorimetric assessment of Oil Red O staining of lipid production. Significant differences in lipid production were observed between PA133 and PA338 (p < 0.01) using Dunn’s multiple comparisons test.
5.3.3.3 Chondrogenesis

Chondrogenesis was quantified using an alcian blue elution assay as described in Chapter 2. Briefly, slides were fixed and stained with alcian blue to demonstrate the presence of GAGs. Alcian blue was then eluted using 0.5ml of 1M HCl, and then quantified using colourimetric detection at 620nm.

Chondrogenesis of pericytes (n=3) was demonstrated in all samples cultured in chondrogenic media on polymer coated coverslips and also control wells with no coating as evidenced by the presence of GAGs and positive blue staining with alcian blue (Figure 5.16 A).

General trends were observed with PA133 and PA337 coated coverslips showed greater amounts of GAG production when compared to other polymers and controls. There were no significant differences observed between the means of the groups when analysed using the Kruskall-Wallis test. Furthermore, no significant differences were observed when the individual groups were compared with each other using Dunn's multiple comparisons test.
Figure 5.16. Chondrogenesis of adipose derived passage 4 and 5 pericytes (n=3) on polymer coated glass coverslips. A.) Appearance of cells following 4 weeks of chondrogenic differentiation on polymer coated glass coverslips (Staining with alcian blue). B.) Quantitative assessment of chondrogenesis using colorimetric assessment of alcian blue staining. No significant difference were observed using Dunn’s multiple comparisons test.

5.4 Polymer analysis

The 5 lead polymers identified through the initial screens were all from a library of co-polymers synthesised from acrylate, acrylamide and vinyl monomers. A summary of the composition of these monomers can be seen in table 5.4.
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<thead>
<tr>
<th>Name</th>
<th>Polymer composition</th>
<th>Ratio (%)</th>
</tr>
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<td></td>
<td>Monomer 1</td>
<td>Monomer 2</td>
</tr>
<tr>
<td>PA133</td>
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<td>GMA</td>
</tr>
<tr>
<td>PA338</td>
<td>MMA</td>
<td>GMA</td>
</tr>
</tbody>
</table>

Table 5.4. The composition and ratio of monomers in the selected polymers.

It can be seen from this table methyl methacrylate (MMA) is present in 3/5 polymers, 2-Methoxyethylmethacrylate (MEMA) and N-methylaniline functionalized glycidyl methacrylate (GMA) are each present in 2/5 polymers with 2-vinylpyridine (VP-2), 4-vinylpyridine (VP-4) and ethylene glycol methacrylate phosphate (EGMP-H) each being present in 1/5 polymers.

Four of the 5 polymers contained monomers with amine groups. VP-2, and VP-4 both contain tertiary amines, whilst MAn which was used to functionalise GMA in PA337 and PA338, contains a secondary amine. These nitrogen containing moieties can form bonds with hydrogen presenting molecules such as water and proteins including integrins which are important in cellular attachment. Amines and the phosphate group contained in EGMP-H are commonly found in the body and therefore these moieties are thought to demonstrate desirable biocompatibility (Table 5.5). Methyl Methacrylate
(MMA) was present in 3/5 polymers. Previous work has demonstrated the methyl (-CH3) functional group to play a critical role in the maintenance of stem cell state in MSC (Curran et al. 2006; Benoit et al. 2008).

<table>
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<tr>
<th>Abbreviation</th>
<th>Full name</th>
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Table 5.5. Full names and chemical structures of the monomers.
5.5 Discussion

Within this chapter a high throughput approach has been utilised which has successfully identified candidate polymers that can be used to support the long term, and stable growth of pericytes, whilst maintaining their phenotype. Polymers were also shown to support osteo-, adipo- and chondro-genesis of pericytes.

Initial screen rapidly identified a small number of distinct polymers that preferentially bound populations of human adipose derived pericytes on repeated testing. The results shown within this chapter suggest that polymer-cell interactions are specific due to the small number of polymers that bound cells compared to the large numbers of polymers that showed little or no binding. This effect may be enhanced by the fact that homogenous populations of purified pericytes were used for the screening thus minimizing the effects of any heterogeneity or contamination within the cell samples. In a similar experiment using heterogenous adipose derived MSC, the number of candidate polymers was significantly larger (Duffy et al. 2014). Further evidence that the polymers identified within this experiment are specific to pericyte binding and function are that they are entirely distinct from the polymers identified when the microarray was used with other distinct cell populations (Hay et al. 2011; Pernagallo et al. 2012).

The effects of polymers on the behavior and function of pericytes was assessed and quantified. All polymers tested were able to support the long
term and stable proliferation of pericytes when assessed using flow cytometry. Growth rates on polymers PA337 and PA338 were greatest and cells appeared to lack the normal cell-cell contact inhibition seen in standard culture. Osteogenic, adipogenic and chondrogenic differentiation was quantified, and although differences were observed between polymers they were not significant. Whilst general trends were observed, differences in the ability of cells to differentiate between different patient samples produced large variation in the results making statistical significance difficult to demonstrate with this relatively small sample size. This has potential clinical implications if that variation limits the ability of individual donors cells to adequately differentiate to the require purpose.

Assessment of the ability of polymers to support chondrogenesis was performed in monolayer cultures. Staining using alcian blue demonstrated the production of GAGs and therefore showed that pericytes are capable of producing cartilage specific ECM. Monolayer culture and quantification of GAG production is not sensitive enough to assess whether enough ECM is produced to engineer biomechanically stable cartilage able to withstand the forces associated with ear reconstruction. Assays that will allow this to be ascertained using 3-dimensional tissue engineering are currently being developed and are discussed further in Chapter 7.
6 Conclusions

The basic principle of plastic and reconstructive surgery is to restore form and function. In doing this, surgeons have to balance the potential benefits against the risks and morbidity associated with the procedure. Auricular reconstruction is an example where the morbidity associated with the harvest of costal cartilage has prompted surgeons to seek for alternatives to the current practice.

Studies using a variety of cells types, biomaterials and fabrication techniques have aimed to generate cartilage de novo for the purposes of auricular reconstruction. Despite much effort, the results have been variable and no techniques are currently being used in routine clinical practice. However, the combination of appropriate stem cells seeded onto a suitable scaffold appears to be the most likely to deliver the solution. Thus the aims of this project were to identify cells and substrates for the purposes of auricular reconstruction.

Mesenchymal stem cells have been studied extensively for their ability to differentiate into mesodermal lineages in vitro (Caplan 2007), and their ability to generate chondrocytes is well established (A. H. Huang et al. 2005). Traditional sources of MSC such as bone marrow are limited in the amount of tissue that can be harvested, and also the morbidity associated with that harvest. Adipose tissue has been shown to be a source of MSC, and in
contrast to bone marrow can be harvested in large quantities with minimal morbidity. Therefore the first section of this thesis sought to establish a tissue bank to collect, store and utilise adipose derived MSC for research.

Prior to establishing the tissue bank we sought to gain an understanding of the attitudes of potential donors toward tissue donation and the use of stem cells in research. Whilst 82% of patients had heard of stem cells, only 4% were aware that adipose tissue was a useful source of stem cells for regenerative medicine. Despite this lack of knowledge, after a simple explanation all patients stated they would be happy to donate tissue. Therefore, despite a prior lack of knowledge, patients undergoing cosmetic lipectomy procedures present as excellent potential donors. Tissue collected through the tissue bank has been used to facilitate the research contained within this thesis and numerous other projects and successful grant applications.

Data from our group and others has demonstrated the ability of pericytes to function as MSC in vitro, and that these cells may represent the in vivo counterpart of the MSC previously observed (Crisan et al. 2008; Covas et al. 2008). These findings were confirmed specifically with pericytes from adipose tissue. Using IHC it is demonstrated that pericytes reside in vivo on the abluminal surface of endothelial cells and can be identified by a unique surface marker profile. They express markers associated with conventional MSC both in vivo and when cultured ex vivo. In addition they can differentiate
into multiple mesodermal lineages when cultured in the appropriate conditions, including chondrogenesis as assessed by staining with Alcian Blue.

This unique surface marker profile can be used to prospectively isolate purified populations of cells using FACS. In addition, adipose tissue contains a second anatomically and phenotypically discrete population of MSC precursor – adventitial cells– together with pericytes they are referred to as perivascular stem cells (PSC). These cells can be purified to homogeneity using flow cytometry in clinically relevant numbers from modest amounts of lipoaspirate thus potentially eliminating the need for purification and expansion by in vitro culture prior to clinical application. The number and frequency of cells are minimally affected by factors such as age, sex, BMI and storage time in this cohort, however further studies are required to examine the effects of extreme age and pathology on the number and efficacy of these cells.

With specific reference to the use of these cells in auricular reconstruction, it is estimated that 50 million cells would be required to generate sufficient cells for clinical use. Based on the data presented within this chapter that would require on average 400cc of lipoaspirate if both PSC populations were to be used, and 1600cc if only pericytes were to be used. Therefore, prospective use of PSC is not a viable option in children where 400cc of lipoaspirate is unlikely to be achievable, and cells expanded in vitro will be required.
Through the use of the high-throughput microarray platform, distinct polymers that support the long term phenotype of pericytes *in vitro* have been identified. Assays that can sensitively quantify the ability of the polymers to support chondrogenesis are actively being developed. In particular assays that allow the quantification of cartilage specific ECM are required to show that the cells are capable of generating cartilage capable of the requirement of *in vivo* implantation and long term survival.

Work that has contributed toward this thesis has led to the establishment of a biobank that has provided cells for this project and numerous collaborations. In addition it has highlighted the need for clinicians and researchers to understand patients attitudes towards research and tissue donation in order to maximise access to tissue. The patients encountered in this study were very willing to donate tissue when provided with the appropriate information.

The ability to purify pericytes from adipose tissue using FACS has been demonstrated, and they have been shown to be a candidate cell source of mesenchymal tissue engineering. The ability to demonstrate their purity and identity has significant advantages over conventional MSC in terms of clinical translation. However, robust evidence that they are able to produce cartilage of the quality required for tissue engineering is lacking.
This thesis provides a detailed analysis of the identity of stem cells within adipose tissue and confirms their niche to be perivascular. It is the largest analysis of the stem cell content of adipose tissue within the published literature, and details the patient and procedure based factors that may influence this yield. This is fundamental information for the clinical translation of these cells.

Distinct synthetic polymers that are able to support the attachment, long term stable proliferation and subsequent differentiation of pericytes have been identified. These polymers have been shown to support this action \textit{in vitro}, and thus provide the basis for the design of scaffolds for \textit{in vivo} implantation.

Collectively this body of work provides the platform on which further studies can be based with the aim of delivering a tissue engineered auricle for reconstructive surgery.
7 Future directions and preliminary studies

7.1 Assays of chondrogenesis and cartilage tissue engineering

Professor Anthony Hollander (Currently The University of Liverpool, previously The University of Bristol)

The most widely used *in vitro* assay of chondrogenesis is the ‘pellet’ in which 300,000 – 500,000 cells are centrifuged to form a pellet which is then placed in chondrogenic differentiation media for a period of 4-6 weeks, as demonstrated in Chapter 4. This assay is believed to replicate the early condensation of mesenchymal precursors in the developing limb buds that subsequently differentiate into cartilage. The pellet assay has been used to demonstrate the ability of stem cells, including MSC and PSC, to differentiate into chondrocytes. Despite this, it is not itself clinically translatable as the conditions in which the assay are performed can not be scaled up to clinically relevant models.

Whilst this assay has been used and replicated in many publications, it has significant limitations and drawbacks. Each pellet requires 300,000 – 500,000 cells, and can only be used for a single type of analysis eg Histology or PCR/qPCR or quantification of protein/ECM production. Furthermore, due to the small amounts of RNA and protein recovered from a single pellet, multiple pellets are required for each assay performed. Experience from this
work found that an average of 8 pellets were required to recover enough RNA to perform basic qPCR experiments. Therefore once replicates are included the number of cells required for a single experiment is very large and makes assessing different factors prohibitively difficult. Therefore, I have sought to learn and develop alternative assays that required fewer cells, were relevant to the ultimate aim of generating tissue engineered cartilage, and allowed quantitative assessment of the amount and quality of cartilage being produced.

Figure 7.1 Protocol for the 3-D chondrogenic differentiation of MSC/pericytes on PGA scaffolds.
Figure 7.2 Discs of neo-cartilage generated from MSC using the protocol described in Figure 7.1.

I had initially planned to combine this technique with the polymers identified in chapter 5 and coat the PGA discs with the polymers and quantify their effects on chondrogenesis. However, the polymers are dissolved in solvents (THF and NMP) and when the PGA discs were placed in the polymer solutions, they dissolved and lost their structure making further assessment impossible. To avoid this issue I aim to develop scaffolds from the polymers alone using 3-D printing technology (detailed in next section).
7.2 Identification of chondrogenic subsets of MSC / PSC

In collaboration with Professor Anthony Hollander – University of Liverpool.

The manuscript generated from this work is currently under review with Science; Translational Medicine.

Cultured MSC are recognized as heterogenous populations of cells. This was clearly demonstrated by Guilak et al who showed that there was marked variation in the ability of different clonal populations of ADSC to differentiate into multiple lineages. Only 52% of clonal populations were able to differentiate into 2 or more lineages, with only 43% demonstrating chondrogenic ability (Guilak et al. 2005).

Surface markers that have been used to define MSC (Dominici et al. 2006) are neither inclusive nor exclusive of the ability to differentiate into mesodermal lineages and therefore are not reliable markers for MSC function. For example, CD105 expression has been shown to be inversely related to osteogenic potential of MSC (Levi et al. 2011). It is therefore recognized that MSC are likely to harbor subsets of cells with specific functions, and the need to identify functional markers of subsets is required. A method of identifying and isolating MSC with an enhanced capacity for cartilage formation would be a useful tool in regenerative medicine.
In an experiment designed to identify markers for highly chondrogenic subsets of MSC, Professor Anthony Hollander and his team produced multiple MSC clones from human bone marrow and quantitatively assessed their ability to differentiate into chondrocytes, produce proteoglycans and type II collagen. mRNA was extracted from the 4 clones that demonstrated the highest and lowest chondrogenic ability and analysed using a gene microarray to identify surface markers that were exclusively expressed by the highly chondrogenic clones. Using this platform, ROR2 - a Wnt5a receptor - was identified as a marker predictive of highly chondrogenic subsets of MSC. Using ROR2 positive cells, Professor Hollander and colleagues have demonstrated enhanced chondrogenesis in vitro, and also in large animal models with 30% more tissue engineered cartilage produced compared to control. They also observed a 70% increase in the production of proteoglycans, and a high type II versus type I and X collagen ratio, which indicates high quality cartilage.

All of the observations so far had been made using cells cultured and expanded in vitro. Professor Hollander asked our group to help determine if ROR2 expression was an artifact of the in-vitro process, or if ROR2 was also expressed natively by MSC. Using techniques identical to those described in Chapter 4 to demonstrate the location and identity of pericytes, I was able to demonstrate the native expression of ROR2. Using immunohistochemistry, ROR2 expression was observed in the developing limb bud and co-localised with MSC markers CD90 and CD105, and pericyte marker CD146. Smaller
numbers of ROR2 positive cells were also seen in perivascular locations in adult bone marrow sections. Using flow cytometry on freshly isolated adipose tissue and bone marrow, ROR2 positive cells were observed. 67% of the CD146+ pericytes in bone marrow were ROR2 positive compared to 19% of the pericytes in adipose tissue. This data confirmed that ROR2 positive MSC precursors are a native cell, and that they are present in more than one tissue type. Whilst the bulk of the work in this thesis used standard pericytes, future work will aim to evaluate the potential of adipose derived ROR2 positive cells for auricular tissue engineering.
7.3 Purification and culture of clinical grade cells

In collaboration with Professor John Campbell, Scottish National Blood Transfusion Service (SNBTS). £12,000 of funding already secured from William Rooney Plastic and Burns Surgery Research Trust.

MSC have been the focus of much attention due to their ability to differentiate into mesodermal tissue, to modulate the immune system and to stimulate regeneration through the secretion of cytokines and trophic support. Through this research 2 anatomically and phenotypically distinct populations of MSC precursors that reside in the walls of blood vessels – namely perivascular stem cells (PSC) have been identified. As detailed in chapter 4, methods for the purification and isolation of these cells using fluorescence activated cell sorting (FACS) have been developed. Using cells isolated by this method our group has demonstrated in a range of in vitro and in vivo assays a function that is equal to, and on occasions superior to standard MSC. The ultimate aim is to translate this pre-clinical research to novel therapies for a range of different treatments including auricular reconstruction.

Whilst FACS allows purification of cells for use in research, there are very few FACS machines that are licensed for use in humans and their cost at present make their use prohibitively expensive. Therefore, to allow translation of pre-clinical research to human therapies, an alternative method of prospective purification needs to be considered. Magnetic cell sorting
(MACS) is similar to FACS and allows specific cells to be purified based on surface marker profile. Magnetic Cell Sorting (MACS) also uses antibodies against specific cell surface markers but the particles are conjugated to paramagnetic particles and not fluorescent particles. Significantly, this technique is licensed for human use with clinical grade antibody conjugates and the CliniMACS separator readily available. This method has been used to purify a variety of different cells used in clinical trials. MACS reagents are available at lab and clinical scale. Therefore, small scale experiments can be directly scaled up to clinical scale. A current collaboration with SNBTS to modify the current FACS based protocols to utilise MACS to derive PSC at clinical grade. If successful, this fundamental step would allow us to translate our research findings to use PSC in clinical trials.

Translation of FACS protocols where populations are excluded by electronic gating, and the selection of specific cells by collection of the desired fraction can be reproduced by magnetic selection by first magnetically depleting the start population of unwanted cells, and then positive enrichment of the desired cells. Our existing FACS protocol excludes CD45+ (haematopoietic) and CD31+ (endothelial) cells, these can be depleted from primary adipose mononuclear cell preparations using biotinylated antibodies to these markers and anti-biotin MACS beads. We will compare the efficiency of depletion using 3.5um MACSi beads and 50nm Microbeads and columns – reagents for both methods are available at clinical grade. PSC will then be isolated using CD146 and CD34 microbeads and the appropriate selection column.
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pp.296–297.


APPENDIX 1

List of book chapters, published work, presentations, prizes and grants related to this work

Book chapters

C.C. West, NS Khan, F. Rossi, M. Crisan
*Methods in Molecular Biology*
Assessment of Pericyte Phenotype by Immunohistochemistry and Flow Cytometry.

CC West

Publications

West CC, Khan NS, Crisan M
*Methods in Molecular Biology (In press)*
Characterisation of Pericyte Phenotype *in vivo* by Immunohistochemistry

NS Khan, West CC, Rossi F, Crisan M
*Methods in Molecular Biology (In press)*
Assessment of Pericyte Phenotype by Flow Cytometry

CC West, WR Hardy, IR Murray, A James, M Corselli, S Pang, C Black, SE Lobo, K Sukhija, P Liang, V Lagishetty, DC Hay, KL March, E Ting, C Soo, B Péault.
*Stem Cells Research and Therapy.* March 2016. 30;7:47.
Purification of perivascular presumptive mesenchymal stem cells from human adipose tissue: process optimization and cell population metrics across a large cohort of diverse demographics.

Pericytes for the treatment of orthopaedic conditions.

Patient’s Attitude towards the Donation and Use of Adipose Tissue and Adipose Derived Stem Cells for Research.

Ethical, Legal and Practical Issues of Establishing an Adipose Stem Cell Bank for Research.

Natural History of Mesenchymal Stem Cells, from Vessel Walls to Culture Vessels

Identification of mesenchymal stem/progenitor cells by flow cytometry.
Presentations

A high-throughput approach to identify defined polymer based substrates for tissue engineering using adipose derived perivascular stem cells
West CC, Murray IR, Stewart KJ, Hay DC, Bradley M, Péault B
European Association of Plastic Surgeons (EURAPS), Edinburgh, 2015.

Perivascular Source of Mesenchymal Stem Cells - the end for culture expansion?
West CC, Murray IR, Hardy WR, Zhang X, James A, Stewart KJ, Ting K, Soo C, Péault B.

The in-vitro effects of synthetic polymers on the behavior of adipose derived peri-vascular stem cells

Adipose derived perivascular stem cells are a source of purified autologous mesenchymal stem cells for regenerative medicine – Is the need for ex-vivo culture over?
West CC, Murray IR, Hardy WR, Zhang X, James A, Stewart KJ, Ting K, Soo C, Péault B.
EURAPS Research Council Meeting. Isle of Ischia, Italy. May 2014.

The effects of synthetic polymer based biomaterials on the behavior of adipose derived perivascular stem cells in-vitro.
International Federation of Adipose Therapeutics and Science (IFATS). New
York. November 2013

Blood, Sweat and Ears

Christopher West

Improvement of fracture healing in atrophic non-union using adipose derived perivascular stem cells.

West CC, Tulyapruek T, B Péault, H Simpson.
European Society of Artificial Organs. Glasgow. September 2013

Therapeutic applications of peri-vascular stem cells (PSC).


Selection and proliferation of adipose derived peri-vascular stem cells (PSCs) using high throughput polymer microarray screening.


Prizes

Winner
B Braun Medical Ltd / Royal College of Surgeons of England Travel Award, Japan, 2016

Best presentation
European Association of Plastic Surgery, Edinburgh, 2015
A high-throughput approach to identify defined polymer based substrates for tissue engineering using adipose derived perivascular stem cells
Winner
The University of Edinburgh 3 Minute Thesis Competition, 2013
“Blood, sweat and ears”

3rd Place
The International Final of the 3 Minute Thesis Competition, 2013
“Blood, sweat and ears”

Best presentation
Scottish National Plastic Surgery Meeting, Dunkeld, 2011
Selection and proliferation of adipose derived peri-vascular stem cells (PSCs) using high throughput polymer microarray screening.

Grants

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<td>William Rooney Plastic Surgery Research Award</td>
<td>2015</td>
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<tr>
<td>University of Edinburgh Travel Award</td>
<td>2013</td>
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<td>Joint Action Research Grant</td>
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<td>Royal College of Surgeons of England Research Fellowship</td>
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<td>Chief Scientist Office Clinical Academic Fellowship</td>
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<td>William Rooney Plastic Surgery Research Award</td>
<td>2011</td>
<td>£22,500</td>
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APPENDIX 2

Collaborations and outputs from the tissue bank

University of Edinburgh

Dr Kate Cameron

Cameron K, Travers P, Chander C, Buckland T, Campion C, Noble B.


Directed osteogenic differentiation of human mesenchymal stem/precursor cells on silicate substituted calcium phosphate.

Professor Hamish Simpson


Adipose derived pericytes rescue fractures from a failure of healing – non-union.

Professor Mark Bradley


Long term mesenchymal stem cell culture on a defined synthetic substrate with enzyme free passaging.

Cairnan Duffy, Rong Zhang, Siew-Eng How, Annamaria Lilienkampf, Guilhem Tourniaire, Christopher C. West, Paul De Sousa and Mark Bradley


A High-Throughput Polymer Microarray Approach for Identifying Defined Substrates for Mesenchymal Stem Cells.

Dr Zoi Michailidou

Diabetes. 2015 Mar;64 (3):733-45.
HIF-prolyl hydroxylase 2 deficiency suppresses murine and human adipocyte lipolysis

Dr Cristina Esteves
Esteves CL, Kelly V, Breton A, West CC, Donadeu FX, Péault B, Seckl JR, Chapman KE.
Pro-inflammatory cytokine induction of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) in human adipocytes is mediated by MEK, C/EBPβ and NF-κB/RelA

University of Glasgow

Professor Matt Dalby
Enateri V. Alakpa, Vineetha Jayawarna, Christopher C. West, Sanne Bakker, Bruno Péault, Karl V. Burgess, Rein V. Ulijn & Matthew J. Dalby.
Tunable Supramolecular Hydrogels for Selection of Lineage-Guiding Metabolites in Stem Cell Cultures

Scottish National Blood Transfusion Service

Professor John Campbell / Dr David Colligan
Mesenchymal stromal cells for co-transplantation with pancreatic islets to improve graft function in type-1 Diabetes.
£225,000 of funding secured.
Isolation of clinical grade PSC from adipose tissue using Magnetic Activated Cell Sorting.
£12,000 of funding secured.

The University of Bristol (now Liverpool)

Professor Anthony Hollander
Sally C. Dickinson, Catherine A. Sutton, Rhys L. Williams, Christopher C. West, Denis Evseenko, Ling Wu, Kyla Brady, Suzanna Pang, Roberta Ferro de Godoy, Allen E. Goodship, Bruno Péault, Ashley W. Blom, Wael Kafienah and Anthony P. Hollander.

Currently under review - Science Translational Medicine

The Wnt5a receptor ROR2 is a predictive cell surface marker of human mesenchymal stem cells with an enhanced capacity for chondrogenic differentiation.

Other groups who have received cells/tissue

Dr Roland Stimson – University of Edinburgh.
Kay Samuels – University of Edinburgh / SNBTS
Dr Dave Hay – University of Edinburgh
Professor Ludovic Vallier – University of Cambridge