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ADIPOSE TISSUE INDUCTION IN VIVO

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26.1. ABSTRACT

Engineering adipogenic tissue *in vivo* requires the concomitant induction of angiogenesis to generate a stable long-term three-dimensional construct. Histioconductive tissue engineering strategies have been used. The disadvantage of using biodegradable scaffolds is a delayed angiogenic induction resulting in ischemic necrosis of the central cell population in the scaffold. We evaluated an histioinductive approach for adipose tissue engineering by combining essential key components for adipogenic induction: (1) a precursor cell source, (2) a vascular pedicle, (3) a supportive matrix, and (4) a chamber to preserve space for the new tissue to develop. We observed concomitant adipogenic and angiogenic induction after 6 weeks in three-dimensional adipose tissue constructs.

26.2. INTRODUCTION

Adipose tissue is a dynamic, easily manipulated tissue which makes it practical for tissue augmentation or contour repair for soft tissue defects of any etiology. Treatment of soft tissue defects is not just a matter of "filling the gap" but of generating a long-term, stable tissue which interacts with adjacent tissue. Problems associated with fat flap transfer, such as resorption at the recipient site and donor site morbidity, are an issue but no ideal surgical alternative or substitutive tissue currently exists.

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Identification of the intrinsic cellular and molecular characteristics of adipose tissue could identify new pathways in tissue engineering research for enhancing fat survival or inducing *de novo* adipogenesis at the recipient site, using either conductive or inductive strategies. Both types of strategies require several key components for a successful construct: progenitor cells, growth factors and cytokines to induce differentiation, an extracellular matrix, a vascular supply and space for the new tissue to develop. Several theories on adipose tissue generation using free fat grafts have been proposed, such as the host replacement theory and the cell survival theory. In the host replacement theory, host derived histiocytes would invade the graft taking on lipid material and replacing all adipose tissue of the graft [1]. In the cell survival theory, histiocytes would act as scavengers of lipid but would not replace the graft adipose tissue. Only part of the graft tissue would survive and be present in the tissue construct after the host reaction subsides.

The extracellular matrix (ECM) plays a number of essential roles in tissue engineering by providing a scaffold for cells to attach and migrate to, as a microenvironment of differentiation, and a preserver of space.

26.3. TISSUE ENGINEERING STRATEGIES

Histioconductive approaches use *ex vivo* biodegradable scaffolds for replacement of missing tissue. Cultured or isolated cells can be seeded onto those scaffolds and implanted *in vivo* [2,3,4]. The substrate dependence of specific cells for proliferation and differentiation will be an essential factor to take into account in this method. On the other hand, the disadvantage here is that pre-cultured tissue constructs, which must become vascularized once implanted within the recipient, may not be as successful in the long-term as methods which foster a primary neovascularisation of a biological matrix scaffold. This is followed by secondary recruitment and migration of native cells with stem cell characteristics for the production of the wanted tissue in an inductive manner. The central cell population of these pre-cultured constructs will be at risk of ischemia before any appropriate vascularisation of the graft occurs.

Growth of any tissue requires *a fortiori* the formation of a functional and mature vasculature. Histioinductive approaches will facilitate self-repair or tissue generation *de novo* at the recipient site. Kawaguchi [5] obtained vascularized plugs of newly formed adipose tissue by injecting Matrigel® supplemented with bFGF into the subcutaneous fat pads of mice. Matrigel® is an extracellular matrix hydrogel derived from the murine EHS sarcoma, and contains basement membrane proteins such as laminin, collagen IV and heparan sulfate proteoglycans as well as several growth factors, such as bFGF, TGF-β, IGF-1, PDGF, NGF and EGF [6]. They further demonstrated that subcutaneous injection of Matrigel® enriched with bFGF, induced neo-adipogenesis in mice [5]. Neovascularisation at the site of injection, together with the basement membrane-rich Matrigel® matrix, apparently creates a suitable micro-environment for endogenous precursor cells to migrate, proliferate and differentiate into mature, vascularized adipocyte clusters [7]. In this *in vivo* model, access to fat is constitutive and adipogenesis is *de facto* host-derived. They suggest that the endogenous progenitor cells penetrated the Matrigel® matrix, in addition to the migration of endothelial cells, from the surrounding host tissue.

In our studies (Cronin et al., 2004; Kelly et al., 2005, Tissue Engineering, on press; Stillaert et al., 2005, in preparation) we used a sealed tissue engineering chamber based

on a vascular pedicle (Figure 26.1.) and supplied with an instructive matrix (BD Matrigel®) and access to adipose tissue. Chambers without a fat xenograft did not generate adipogenic tissue. The rationale of transplanting a fat tissue graft in a suitable ECM was that early studies using the closed chamber showed that access to preexisting adipose tissue was essential (Kelly et al., 2005) to generate adipogenic tissue. The cell-cell or cell-matrix interactions could be temporarily better preserved and cells in whole tissue biopsies could resist hypoxic conditions longer when placed in Matrigel®. We initially hypothesized that the vascularized fat tissue generated in the chamber (Figure 26.2.) originated from the adipose tissue graft, more specifically from its stromal-vascular fraction located precursor cell population. However, careful immunohistochemical analysis of human xenografted fat biopsies in SCID-mice revealed the generated fat tissue was predominantly host-derived. An alternative hypothesis was that those isolated fat sources may be providing stimuli for the recruitment of mesenchymal stem cells directly from perivascular cell populations within the chamber or from the systemic circulation via the newly developed vascular network (Figure 26.2.).

These studies have considerable bearing on the growing number of approaches to adipose tissue engineering, such as use of inductive fat with autografted "preadipocytes" or preadipocyte conferring components such as processed-lipoaspirate (PLA). It may also shed further light on the question of whether it is graft survival ("cell survival theory") or *de novo* adipogenesis ("host replacement theory") or a combination of both which occurs during autologous fat transplantation, with significance for trying to understand why such variable results are obtained with this widely utilized technique.

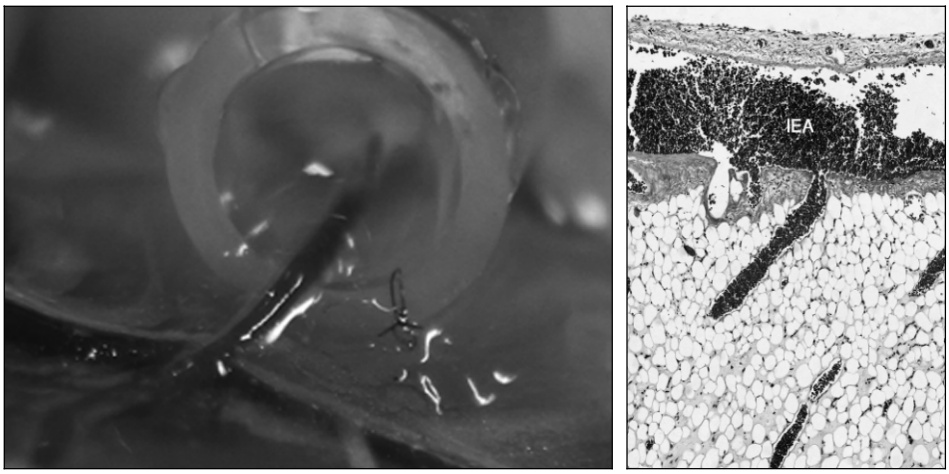

Figure 26.1. Left. Silicon tissue engineering chamber positioned around the inferior epigastric artery ("IEA") in the mouse groin. **Right.** Angiogenesis sprouting from the main vascular pedicle (IEA) with concomitant adipogenesis (magnification × 20).

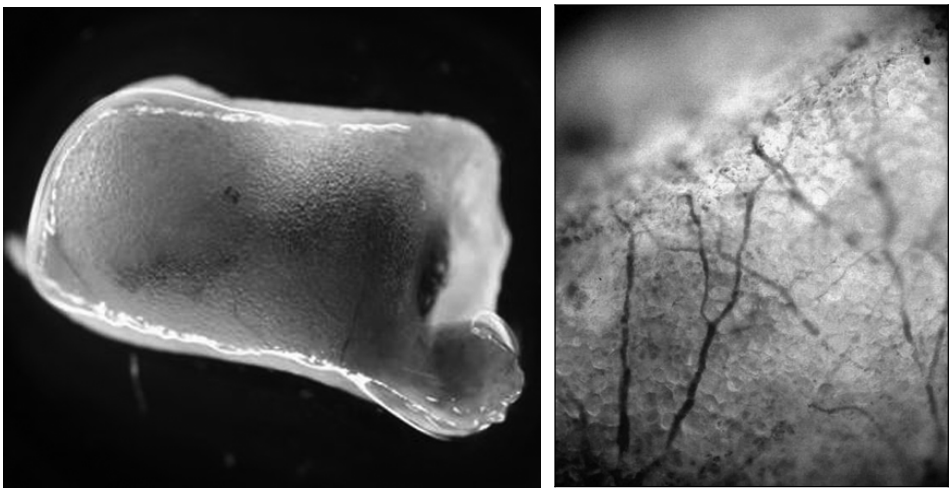

Figure 26.2 Left. In vivo generated 3D fat construct after 6 weeks. **Right.** Angiogenesis at the surface of the generated fat construct resulting in a 3D vascular framework supporting further adipogenic tissue development.

26.4. POTENTIAL PROGENITOR CELLS FOR ADIPOSE TISSUE ENGINEERING

Within the connective tissue matrices of most adult organs there are lineage-committed and lineage-uncommitted progenitor cells able to differentiate towards different cell lineages under appropriate differentiation conditions [8]. Adipose tissue is derived from the embryonic mesoderm, and contains a heterogeneous stromal–vascular fraction (SVF) which includes such progenitor cells.

Isolated lineage-uncommitted stromal cells from subcutaneous fat have been shown to be capable of differentiating *in vitro* into adipocytes and other cell types when cultured in the presence of established differentiation factors [8]. Others have used exogenous SV fraction/preadipocytes to induce fat, usually subcutaneously [9]. This population in the SVF is phenotypically similar to mesenchymal stem cells (MSCs), they express some of the CD antigens observed on bone marrow MSCs but they also exhibit an unique CD marker profile and gene expression, distinct from those seen in MSCs [10]. Adipose precursor cells in developing fat pads arise from multipotential MSCs, whose origins are unknown. These stem cells develop into unipotential adipoblasts, which become committed to the adipocyte lineage under the influence of various factors such as hormones, growth factors, cell-cell and cell-matrix interactions which have not yet been fully elucidated, developing into preadipocytes with a fibroblast-like morphology. Although adipoblasts are assumed to appear primarily during embryonic development, it is not clear whether some remain postnatally or whether only preadipocytes are present in the latter stages of development.

Preadipocytes express early differentiation markers such as Pref-1 (Sul et al., 1989) but have not accumulated intracellular triglyceride droplets. Preadipocytes have been estimated to represent 0.02% of the total cell population in the SVF of subcutaneous fat pads in adults [11] and considerable expansion of this population would be desirable for use

in tissue engineering applications. The precursor population within the SVF is heterogeneous, composed of preadipocytes at various stages of differentiation (early, late and very late), and adipoblasts [12]. In our *in vivo* experiment we have included some tissue engineering chambers in which muscle xenografts were implanted. Adult skeletal muscle also contains progenitor cells, known as satellite cells that have been shown to be pluripotent (Asukara et al., 2001; Shefer et al., 2004). A second population of potential progenitor cells in muscle is the muscle derived stem cells which are distinct from satellite cells. The prevailing hypothesis is that both populations co-exist as distinct stem cell tiers in a state of equilibrium within adult muscle, with both cell types having the potential to be used as progenitor cells for adipocytes and adipose tissue engineering. The signaling interaction between myogenic cells and adipocytes has been implicated as playing a significant role in the rate and extent of adipogenesis, myogenesis, and lipogenesis/lipolysis. Key factors in these processes include leptin, insulin-growth factors, and adiponectin [13]. The skeletal muscle-containing tissue engineering chambers did generate a vascularized adipose tissue construct after 6 weeks. In some harvested tissue constructs we observed additional myogenesis *de novo* and this phenomenon was dependant on the presence of a healthy fraction of interstitial tissue within the implanted muscle xenograft (Figure 26.3.).

There is also potential for the recruitment of bone marrow-derived mesenchymal stem cells to our *in vivo* engineered construct. However, little evidence exists so far to support a functional role for circulating cells in mesenchymal tissue repair [14]. The idea is that undifferentiated MSCs, following delivery and migration to the engineered environment, will differentiate into adipocytes under the influence of local signals. The local factors directing this multi-step process are not yet fully determined although several candidates exist, such as leptin (Aprath-Husmann et al., 2001), adiponectin (Farmer, 2005), plasminogen activation inhibitor-1 (PAI-1) [15,16], CCAAT/enhancer binding protein-β (C/EBPβ) (Loftus et al., 1997), PPARγ (Cowherd et al., 1999; Farmer, 2005), hypoxia and insulin.

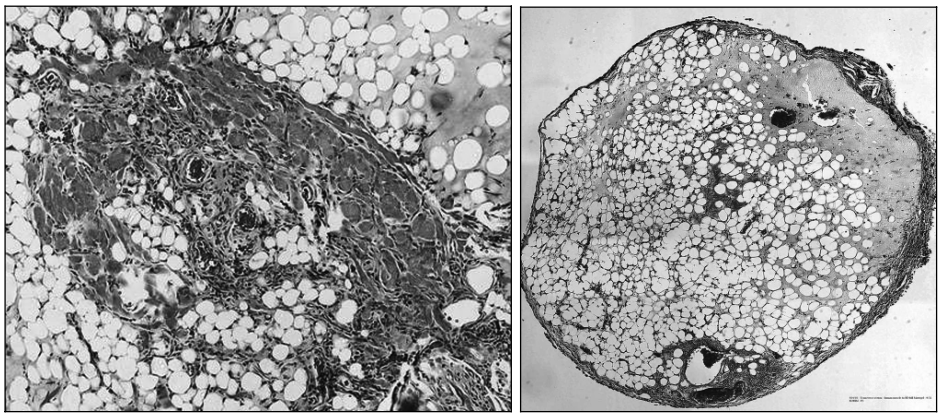

Figure 26.3. Skeletal muscle xenograft implanted in the in vivo tissue engineering chamber. Adipogenic and myogenic induction after 6 weeks (magnification ×20). **Right** Cross section through harvested specimen after 6 weeks containing muscle xenograft in the center. Abundant adipogenesis is observed (magnification ×10)

26.5. EXTRACELLULAR INDUCING SIGNALS

Preadipocytes are capable of synthesizing PAI-1, a key factor in angiogenesis, cell migration differentiation and proteolysis [15,16]. PAI-1 synthesis is increased during preadipocyte migration towards adipose tissue development, and the levels decrease *in vitro* when a confluent preadipocyte layer is attained and active migration ceases. Other key regulatory events in fat cell differentiation include the induction of CCAAT/enhancer binding proteins -β (C/EBPβ) and -δ (C/EBPδ) followed by induction of PPARγ and C/EBPα, which upregulate adipose functional genes. PPARγ appears to be crucial for adipocyte differentiation, with studies showing that blocking the PPARγ pathway in preadipocytes not only inhibits their differentiation into mature adipocytes, but can also inhibits angiogenesis *in vivo* [17].

Preadipocytes have the unique ability to enhance *in vivo* angiogenesis and cause the remodeling of vessels into an efficient network with a mature, stable architecture [18]. Hypoxia is known to be one of the strongest stimuli that boosts capillary angiogenesis and exerts its effect through an upregulation of vascular endothelial growth factor (VEGF). As the PPARγ gene expression is inhibited by hypoxia, angiogenic vessel remodeling may accelerate adipogenesis by increasing hypoxia inducible factor 1 (HIF-1) degradation, thus potentiating PPARγ activation. VEGF is an endothelial mitogen and chemokine, and is highly expressed in adipose tissue, increasing during adipocyte differentiation [19,20,21]. Adipose stromal cells, isolated from human subcutaneous fat tissue, were found to secrete 5-fold more VEGF when cultured in hypoxic conditions. Conditioned media derived from hypoxic adipose stromal cells significantly increased endothelial cell growth with reduced endothelial cell apoptosis. Established preadipocytes in the stromal compartment of white adipose tissue are less vulnerable to hypoxemia. Hypoxia is known to occur in our tissue engineering chambers, transiently (Lokmic et al., in preparation), and this could help drive the adipogenic result..

26.6. EXTRACELLULAR MATRICES AND SCAFFOLDS

The ECM is a non cellular substance, made up of protein and long-chain sugars (polysaccharides) in which cells are embedded. This “biological glue”, in which growth factors can be released from matrix storage, functions as a framework for physical cell support, coordinates cell development via cell-cell and cell-matrix interactions, and in turn stimulates cells to produce the ECM components [22,23]. ECM substrates not only provide mechanical support for cells, but also orientate and constrain cells during regeneration. They provide extra space for the coordination of growth factor and cell derived signals between the ECM and cells, effect intercellular communication, and mediate cellular growth, differentiation and ultrastructural stability [24]. This suggests that binding and storage of growth factors by the matrix are important determinants in regulating cellular metabolism. ECM proteins coordinate cell migration, proliferation and tissue homeostasis by binding to specific integrin cell surface receptors. Binding to those receptors activates intracellular signaling pathways, causing cytoskeletal reorganization and alteration of cell morphology [24]. Cell migration and tissue remodeling events are regulated by different proteolytic systems. For example, degradation of the basement membranes surrounding the capillary is necessary for sprouting angiogenesis to proceed.

Cellular differentiation of new tissues is induced by cues in the microenvironment immediately surrounding cells. Salasznyk et al. (2004) indicates that ECM stimuli also play an important role in inducing osteogenesis of human MSC.

The differentiation of fat precursor cells will depend on spatially and temporally controlled expression of multifunctional adhesive glycoproteins and their cellular receptors, and on a tight regulation of different proteolytic enzyme families. Human preadipocytes accumulate lipid droplets in their cytoplasm and express positive immunoreactivity for collagen type IV and laminin from the 6th week of gestation onward [23]. Culture dishes coated with Matrigel® promote attachment and spreading of preadipocytes whereas spreading of nonpreadipocytes was antagonized. Components, such as laminin which also enhance selective proliferation of preadipocytes [22], play active roles which extend to developmental as well as regenerative processes. This selective spreading of preadipocytes on Matrigel® coated wells has been observed in our *in vitro* experiment with ongoing proliferation of fibroblast-like cells from adult fat biopsies (Figure 26.4.).

Multifunctional glycoproteins are present in the ECM which regulate adhesive processes, coordinating proteolytic degradation and influencing cell migration, proliferation and differentiation. One of those glycoproteins is vitronectin which has been discovered only recently in the SVF of white adipose tissue. It is well-known to play a leading role in cell migration with subsequent differentiation [25], and may well be important in adipogenic tissue engineering.

26.7. VASCULATURE

There is convincing evidence of autocrine/paracrine or developmental relationship between capillaries/endothelial cells and preadipocytes, with vascular cells expressing receptors for most adipocyte-derived factors [26,27]. Adipose tissue and vasculature reside in a steady-state balance with each other and the complex relationship between adipose tissue formation, angiogenesis, and vessel remodeling may explain why isolated fat graft transplantation results in poor and unpredictable results.

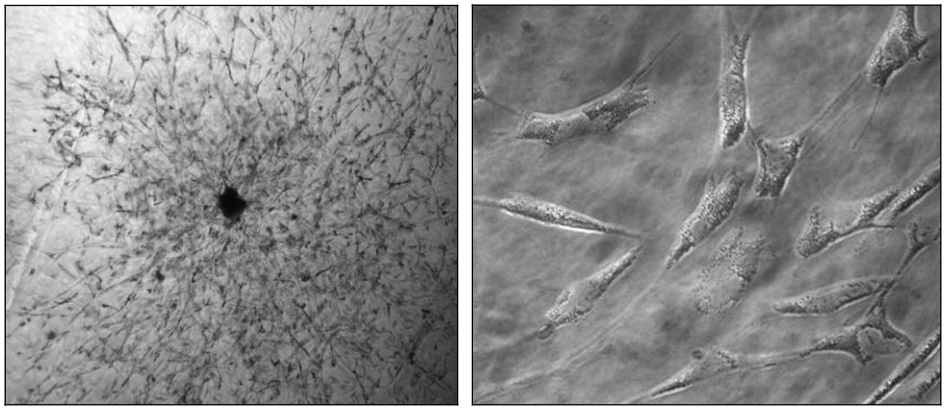

Figure 26.4. Human fat biopsy seeded on Matrigel® coated tissue culture wells. Selective spreading of preadipocytes is observed from the biopsy.

Preadipocytes produce PAI-1 which would ensure coordination of adipogenesis and angiogenesis at the local level [19]. Studies have shown that human preadipocytes and endothelial cells express $\alpha_V\beta_3$ integrin and express and secrete PAI-1, which regulates preadipocyte and endothelial cell migration *in vivo* [19]. Microvascular endothelial cells secrete factors and ECM components which induce proliferation with subsequent differentiation of preadipocytes and neovascularisation will not be triggered without adipocyte differentiation. The established adipose tissue mass in adult life can be regulated through its vasculature, as a wide range of vasoactive signals are secreted by adipose tissue, specifically from the SVF. The expression of factors such as angiopoietins and PAI-1 have been reported, depending on the state of cell differentiation, site of growth, and external stimuli [18]. The molecular mechanisms underlying blood vessel maturation during *de novo* adipose formation are yet to be determined. The close relationship between developing adipocytes and neocapillaries could be observed in our specimens harvested from the sealed tissue engineering after 6 weeks (Figure 26.5.).

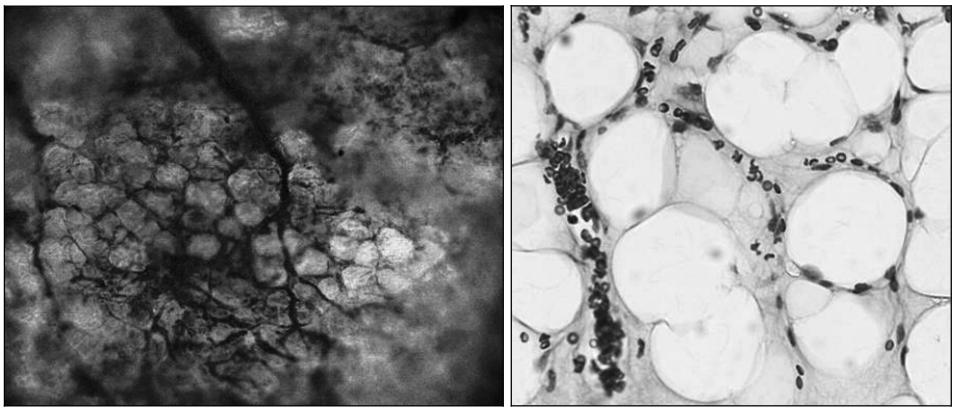

Figure 26.5. Left. Angiogenesis observed at the surface of the harvested fat construct. Adipocytes are surrounded by capillaries. **Right.** Histological section showing adipogenesis and concomitant angiogenesis in the Matrigel® matrix after 6 weeks in vivo (magnification × 60).

26.8. CONCLUSIONS

Engineering fat tissue *in vivo* is a challenging research area as several key factors need to be considered. Not only potential precursor cells but the ECM will play a crucial role as this extracellular compartment will coordinate and regulate ongoing cellular processes. Histioconductive methods with the use of biodegradable rigid scaffolds will be difficult to extrapolate in a clinical setting as the induction of angiogenesis *de novo* will be necessary for further (precursor) cell support and the differentiation into mature adipocytes. Adipose tissue is a densely vascularized tissue and the relationship between those two compartments is fundamental for further stabilization of the generated adipose tissue construct within adjacent anatomical structures.

In this respect, histioinductive methods are preferable as they could foster a complementary and harmonious development of angiogenesis and adipogenesis. The interaction between those two cellular events will depend on the availability of an

appropriate supporting ECM. A human derived supporting ECM is not yet available but our work with the murine derived Matrigel®, a basement membrane substrate, could direct future work in the field of adipose tissue engineering. We observed that xenografts where a considerable fraction of SVF was included generated considerable adipogenesis *de novo* and *in vivo* in our tissue engineering chamber, however the resultant adipogenic induction seemed to be host-derived. The host-derived nature of the adipose tissue construct was rather surprisingly as we observed ongoing proliferation of fibroblast-like cells out of fat biopsies seeded on Matrigel® coated wells *in vitro*. The hypothesis is that signals or cytokines present in the SVF of the implanted xenografts could direct the adipogenic processes in the tissue engineering chamber and this particular fraction will be the subject of future research in order to identify those cytokines responsible for inducing adipogenesis at the recipient site. Subsequently, this could enhance the development of a human ECM suitable for supporting adipogenesis *in vivo* in a clinical setting.

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