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Adipose-derived stromal cells mediate *in vivo* adipogenesis, angiogenesis and inflammation in decellularized adipose tissue bioscaffolds

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ABSTRACT

Decellularized adipose tissue (DAT) has shown promise as an adipogenic bioscaffold for soft tissue augmentation and reconstruction. The objective of the current study was to investigate the effects of allogeneic adipose-derived stem/stromal cells (ASCs) on *in vivo* fat regeneration in DAT bioscaffolds using an immunocompetent rat model. ASC seeding significantly enhanced angiogenesis and adipogenesis, with cell tracking studies indicating that the newly-forming tissues were host-derived. Incorporating ASCs also mediated the inflammatory response and promoted a more constructive macrophage phenotype. A fraction of the $CD163^{+}$ macrophages in the implants expressed adipogenic markers, with higher levels of this "adipocyte-like" phenotype in proximity to the developing adipose tissues. Our results indicate that the combination of ASCs and adipose extracellular matrix (ECM) provides an inductive microenvironment for adipose regeneration mediated by infiltrating host cell populations. The DAT scaffolds are a useful tissue-specific model system for investigating the mechanisms of *in vivo* adipogenesis that may help to develop a better understanding of this complex process in the context of both regeneration and disease. Overall, combining adipose-derived matrices with ASCs is a highly promising approach for the *in situ* regeneration of host-derived adipose tissue.

1. Introduction

Decellularized bioscaffolds have generated great interest in the field of tissue engineering due to their potential to enhance the regeneration and repair of damaged tissues [1–3]. Ideally, the decellularization process should be designed to gently extract cellular components, while conserving the complex structure and composition of the extracellular matrix (ECM) [4,5]. These strategies can be used to create off-the-shelf biomaterial scaffolds enriched in collagens and other structural ECM components, which can support cell attachment and infiltration both *in vitro* and *in vivo* [6–8]. Importantly, decellularized tissue bioscaffolds can also mimic the tissue-specific biochemical and biomechanical properties of the native cellular microenvironment, which are recognized as key mediators of a broad range of cell functions including proliferation and differentiation [9–11].

In the context of plastic surgery, decellularized adipose tissue (DAT) is emerging as a promising alternative or supplement to fat grafting for soft tissue augmentation and reconstruction [12–14]. In previous work, our group has pioneered a detergent-free adipose tissue decellularization protocol for generating scaffolds from human adipose tissue, which is abundantly discarded as surgical waste [12]. Following decellularization, we have shown that the DAT processed with our methods retains ECM components including collagen I, IV, and laminin [12], and has similar mechanical properties to native human fat [15]. Further, we have also demonstrated that the DAT bioscaffolds have adipo-conductive and adipo-inductive properties, providing a highly supportive microenvironment for the adipogenic differentiation of human adipose-derived stem/stromal cells (ASCs) [12,16–18].

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Our group and others have begun investigating the mechanisms involved in fat formation in adipose-derived matrices, including other bioscaffold formats such as DAT microcarriers [16], porous foams [14,17], injectable gels [19], and hydrogel composites [18]. *In vivo* studies using rodent models have demonstrated that DAT scaffolds provide a supportive microenvironment for soft tissue regeneration, stimulating blood vessel formation and adipocyte development [19,20]. These effects may be mediated by the presence of growth factors or other bioactive components within the decellularized ECM [21]. For example, the angiogenic growth factor VEGF has been identified in human DAT by immunohistochemistry (IHC) [13], and varying levels of VEGF, bFGF, PDGF and TGF-β have been reported in ELISA analyses of decellularized porcine adipose-derived ECM [22,23].

While applying the DAT as an off-the-shelf bioscaffold for adipose reconstruction is attractive from a translational perspective, localized tissue regeneration may be enhanced by incorporating exogenous regenerative cells, such as ASCs, in the decellularized ECM. In a recent study by Young et al., angiogenesis and adipogenesis were stimulated when adipose ECM hydrogels were combined with human ASCs in an athymic mouse model [19]. Similarly, Poon et al. observed enhanced fat formation in their adipose-derived matrix when it was seeded with allogeneic ASCs in a rat model [23]. Based on these previous studies, a cell-based approach may augment the rate of regeneration, which could be advantageous for the reconstruction of larger soft tissue defects.

Although the roles of endogenous ASCs in the context of adipose tissue accumulation and homeostasis have been extensively studied in the field of obesity research, far less is known about the mechanisms of ASC-mediated adipose regeneration in a tissue engineering context. In previous studies, it is often unclear whether delivered ASCs support *in vivo* adipogenesis through direct differentiation into mature adipocytes or if they may enhance fat formation predominantly via indirect paracrine effects. In particular, ASCs can secrete a broad array of cytokines and growth factors that promote angiogenesis, enhance endogenous stem cell recruitment, and modulate the inflammatory response, which may help to co-ordinate *in situ* tissue regeneration [24]. With emerging evidence that macrophages play an important role in adipose tissue regeneration [25,26], it is particularly notable that ASCs can secrete anti-inflammatory cytokines that may promote a more constructive macrophage phenotype [27]. Moreover, this response may be enhanced if DAT is applied as the ASC delivery platform, as other decellularized bioscaffolds have been reported to induce M2 macrophage polarization [28,29].

Overall, the primary objective of the current study was to conduct an in-depth analysis of the effects of ASC seeding of DAT bioscaffolds on adipogenesis, angiogenesis and macrophage response following subcutaneous implantation in an immunocompetent Wistar rat model. DAT samples were seeded with ASCs isolated from male donor rats and implanted into female rats to enable long-term cell tracking via the Y chromosome through fluorescence *in situ* hybridization (FISH) analysis. ASC-seeded and unseeded control scaffolds were explanted at 72 h, 1, 4, 8 and 12 weeks for semi-quantitative histological and IHC analyses of the remodeling implant regions.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals were purchased and used as received from Sigma Aldrich Canada Ltd. (Oakville, ON, Canada).

2.2. Adipose tissue processing and decellularization

For DAT scaffold fabrication, subcutaneous adipose tissue samples were collected from female patients undergoing elective breast reduction or abdominoplasty surgeries at the Kingston General Hospital or Hotel Dieu Hospital in Kingston, Canada, with approval from the Research Ethics Board at Queen's University (REB# CHEM-002-07). The samples were immediately transported to the lab in cold sterile phosphate buffered saline (PBS) with 2% bovine serum albumin (BSA). Adipose tissue decellularization was performed following published protocols [12], and the prepared DAT was decontaminated through repeated rinsing in 70% ethanol, followed by rehydration in sterile PBS supplemented with 1% antibiotic-antimycotic solution (Life Technologies, Burlington, Canada) [12]. The DAT was stored for approximately 1 week at 4 °C prior to scaffold seeding.

2.3. Rat ASC isolation and culture

All studies involving animals followed the Canadian Council on Animal Care (CCAC) guidelines and were reviewed and approved by the Animal Care Committee at Queen's University (Protocol # Flynn-2009-059 & Flynn-2010-053). ASCs were isolated from male rats to enable tracking of the donor cells via the y-chromosome following implantation in female rats, with the epididymal fat pad providing a sizable depot for cell isolation. Methods for extracting and culturing the rat ASCs were based on published human protocols [30]. In brief, male Wistar rats (10–12 weeks in age) were euthanized by CO_2 overdose and the epididymal fat pads were excised and finely minced. The minced tissue was digested in Kreb's Ringer Buffer solution supplemented with 2 mg/mL collagenase (Worthington Type I, Cedarlane, Burlington, Canada), 1% BSA, 2 mM glucose, and 25 mM HEPES at 37 °C for 45 min. The tissue digest was filtered through a stainless steel mesh and the mature adipocytes were removed following gravity separation. The sample was centrifuged (1200 × g, 5 min) and the cell pellet was incubated in erythrocyte lysis buffer (0.15 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA) for 10 min at room temperature. The cell suspension was then filtered through a 40 micron cell strainer to remove debris, and the remaining cells were washed twice and plated in T75 flasks (Corning, Fisher Scientific, Ottawa, Canada) in complete medium comprised of DMEM:Ham's F12 supplemented with 10% fetal bovine serum (Thermo Scientific HyClone, Fisher Scientific, Ottawa, Canada) and 100 U/mL of penicillin and 0.1 mg/mL streptomycin (1% pen-strep) (Life Technologies, Burlington, Canada). Cultured ASCs (37 °C, 5% CO_2) were passaged at 70% confluence and passage 2 (P2) cells were used for the study. To characterize the P2 rat ASCs, we performed immunophenotype analysis using flow cytometry to assess the expression of CD90, CD29, CD44H, CD31 and CD45, and confirmed adipogenic differentiation capacity through *in vitro* cell culture assays (Supplementary Fig. 1).

2.4. Scaffold seeding and implantation

To prepare the scaffold samples for implantation, the DAT was cut into 150 mg ± 10 mg pieces under sterile working conditions and placed in 12-well tissue culture plates. The samples were rinsed twice and then incubated overnight (37 °C, 5% CO_2) in complete medium. For the seeding protocol, the scaffolds were transferred into 12-well tissue culture inserts (0.4 μm pore size; Greiner Bio-One, North Carolina, USA) and seeded at a density of 1×10^6 cells/scaffold in 500 μL of complete medium, with 1.5 mL of complete medium added to the well below the membrane. Unseeded DAT scaffolds were similarly processed as controls. Both scaffold groups were cultured in complete medium (37 °C, 5% CO_2) for 72 h prior to implantation.

The scaffolds were implanted subcutaneously in female Wistar rats following established protocols [17]. A total of 20 rats were