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# Alterations of MEK1/2-ERK1/2, IFNγ and Smad2/3 associated Signalling pathways during cryopreservation of ASCs affect their differentiation towards VSMC-like cells

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ABSTRACT

Vascular smooth muscle cells (VSMCs) play essential roles in regulating blood vessel form and function and they are required for vascular tissue regeneration. Multipotent adipose derived stromal cells (ASCs) can be differentiated into VSMC-like cells, which can be used as a potential VSMC source for the development of vascular tissue. However, the effects of cryopreservation on the differentiation of ASCs towards VSMCs are poorly studied to date. This study compared fresh ASCs (FA) vs. cryopreserved ASCs (CA) with respect to their differentiation potential towards VSMC-like cells. The expression of contractile VSMC markers (such as smoothelin) and cell contractility were investigated. It was found that VSMC-like cells derived from CA expressed smoothelin gene and protein at lower levels and showed compromised contractility in response to vasoconstrictors, when compared with those derived from FA. Moreover, it was demonstrated that this negative effect of cryopreservation could be mediated by MEK1/2-ERK1/2, IFNγ and Smad2/3 associated Signalling pathways. Treatment of CA with MEK1/2-ERK1/2 activator or IFNγ neutralizing antibodies enhanced Smad2/3 phosphorylation and showed a rescue of the negative effect of cryopreservation on the differentiation of ASCs towards VSMC-like cells. These findings are important for defining approaches that may use cryopreserved ASCs for vascular tissue regeneration.

## 1. Introduction

Cardiovascular disease is a leading cause of death globally (Pashneh-Tala et al., 2015). A major fraction of cardiovascular diseases is associated with coronary artery or peripheral artery diseases, which are caused by the blockage of arteries following plaque accumulation (Byrne et al., 2014). In order to restore normal blood perfusion in the narrowed vasculature, the gold-standard treatment is to use an autologous vascular graft to replace the occluded vessel (Collins et al., 2008). However, access to autologous vascular grafts is often limited due to the patients' lower extremity complications or previous blood vessel harvest (Pashneh-Tala et al., 2015; Veith et al., 1979). Over the past few years, tissue engineered vascular grafts (TEVGs), which often combine degradable biomaterial scaffolds and vascular cells, have shown promise as alternatives to the limited supply of autologous blood vessels in order to replace diseased coronary and peripheral arteries (Wang et al., 2010a, 2010b; Wang et al., 2013; Zhou et al., 2016).

Vascular smooth muscle cells (VSMCs) are the predominant cell type that constitute the medial layer of the blood vessels in the body, and assume important structural and physiological functions (Lacolley et al., 2012; Lilly, 2014). During development, VSMCs can produce different types of extracellular matrix molecules such as collagen, elastin, fibronectin and glycosaminoglycan, which can support the structural integrity of the vasculature (Lacolley et al., 2012). In most

*Abbreviations:* ASC, Adipose derived stromal cells; α-SMA, α-smooth muscle actin.; CA, Cryopreserved ASCs.; FA, Fresh ASCs.; IFNγ, Interferon gamma.; NE, Norepinephrine.; RA, Retinoic acid.; SM22α, Smooth muscle 22α.; SMMHC, Smooth muscle myosin heavy chain.; TEVG, Tissue engineered vascular grafts.; TNFα, Tumor necrosis factor alpha.; TGF-β1, Transforming growth factor-β1.; UASMC, Umbilical artery smooth muscle cells.; VSMC, Vascular smooth muscle cells.; Vaso, Vasopressin.

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healthy mature blood vessels, VSMCs typically switch from a synthetic phenotype to a contractile phenotype and they are key players in regulating blood pressure (Brozovich et al., 2016; Lacolley et al., 2012), redistributing blood flow (Brozovich et al., 2016; Lacolley et al., 2012) and maintaining vascular homeostasis (Bennett et al., 2016; Ji et al., 2016; Lilly, 2014). Hence, obtaining VSMCs from an autologous source that can switch to a contractile phenotype is essential for the development of functional and patient-specific TEVGs (Neff et al., 2011). Unfortunately, a significant challenge that the current TEVG field is facing is that autologous VSMCs from mature vessels are difficult to access and have a limited expansion ability in vitro (de Villiers et al., 2009). As a result, mature VSMCs derived from patients' blood vessels are not practical sources of VSMCs for the regeneration of vascular grafts that would eventually be applied in a clinical setting.

Multipotent adipose-derived stromal cells (ASCs) have attracted great interest in the tissue regeneration field because they can be readily obtained by low-risk and well-tolerated liposuction surgeries and have low risks of mutations, epigenetic abnormalities and tumorigenicity (Bajpai and Andreadis, 2012; Rosenbaum et al., 2008), in comparison with other types of stem/stromal cell sources. Previous studies have shown that ASCs can be differentiated towards VSMC-like cells using different biochemical induction factors, co-culture, biomechanical stimulation and biomaterial cueing (Zhang et al., 2017). Additionally, it has been demonstrated that a vascular smooth muscle tissue-like layer pre-developed in vitro using VSMC-like cells derived from ASCs could enhance the integration and functionality of the TEVG after it was implanted in vivo (Neff et al., 2011). Overall, ASCs represent a promising alternative cell source for vascular tissue regeneration.

As tissue regeneration, stem cell research and cell therapy fields have advanced in recent years, there has been a growing body of research on the cryopreservation of ASCs (Minonzio et al., 2014; Thirumala et al., 2010; Yong et al., 2015). The fundamental goal of cryopreservation is to preserve the functionality of ASCs and potentially use such cells in a more efficient and on-demand manner for clinical applications, without the need for repetitive liposuction surgeries. However, current ASC cryopreservation studies have mainly focused on investigating the effect of cryopreservation on the ASC immunophenotype (De Rosa et al., 2009; Yong et al., 2015), proliferation (De Rosa et al., 2009; Minonzio et al., 2014) and tri-lineage differentiation (adipogenic, osteogenic and chondrogenic) (De Rosa et al., 2009; Minonzio et al., 2014; Thirumala et al., 2010; Yong et al., 2015). In addition, the majority of the published investigations relating to the differentiation of ASCs towards VSMC-like cells have only used ASCs freshly isolated from patients' fat tissue and have not critically explored cryopreserved cells (Lee et al., 2012; Merfeld-Clauss et al., 2014; Park et al., 2013; Wang et al., 2010a). There is no existing study known to the authors that has compared the VSMC-like cell differentiation ability of ASCs (isolated from the same donor), simultaneously with and without cryopreservation. Whether ASC cryopreservation (cell freezing and thawing) would have any compromising effects on the specific VSMC gene and protein expression and contractility function of the VSMC-like cells derived from ASCs remains unknown.

Therefore, this study was designed to methodically compare fresh and cryopreserved ASCs (FA and CA, isolated from the same donors) with respect to their differentiation potential towards VSMC-like cells. Specifically, the expression of a defined set of VSMC genes (early-stage markers: α-smooth muscle actin (α-SMA), smooth muscle 22α (SM22α); mid-stage markers: calponin, caldesmon and late-stage markers: smooth muscle myosin heavy chain (SMMHC), smoothelin (Zhang et al., 2017)) and proteins (α-SMA, calponin, SMMHC and smoothelin) and cell contractility in response to vasoconstrictors (norepinephrine (NE) and vasopressin (Vaso)) were investigated in VSMC-like cells derived from fresh and cryopreserved (frozen and then thawed) ASCs. The differentiation of ASCs towards VSMC-like cells is often mediated by the activation of MEK/ERK, p38, JNK, RhoA, Smad2/3 Signalling pathways (Zhang et al., 2017) and the activation of those pathways could be altered as a result of cell cryopreservation (Bissoyi et al., 2014; Cowan and Storey, 2003; Liu et al., 2000; Xu et al., 2010). As a result, the total and activated Signalling proteins (ERK1/2, p38, JNK, RhoA, Smad2/3) were quantified in both fresh and cryopreserved ASCs in this study. Additionally, the release of pro-inflammatory cytokines interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) was also studied in fresh and cryopreserved ASCs, as both can be released by cells in response to cryopreservation (Campbell et al., 2009; Francois et al., 2012) and interact with Smads to potentially regulate VSMC differentiation (Hu and Ivashkiv, 2009; Verrecchia et al., 2000; Wen et al., 2004). Findings related to the effect of ASC cryopreservation on the cells' potential to differentiate towards VSMC-like cells and the Signalling mechanisms delineated in this study will provide significant insight into ASC banking and the application of ASCs for vascular tissue regeneration, potentially avoiding the need for multiple surgical procedures.

## 2. Materials and methods

All chemicals were purchased from Sigma-Aldrich unless stated otherwise.

### 2.1. ASC isolation

ASCs were isolated from fat tissues donated by patients undergoing liposuction surgeries at Toronto General Hospital (University of Toronto ethics approval #13–6437-CE). The fat tissue (approximately 15 mL) was washed with PBS and digested with a collagenase digest solution (23.6 mL Krebs-Ringer Bicarbonate solution, 1.43 mL 35% BSA solution, 3 mM glucose, 2 mg/mL collagenase type II and 25 mM HEPES) at 37 °C for 45 min (with continuous agitation at 100 rpm). The digested sample was filtered through a 250 μm pore filter to remove any undigested tissue segments. The floating adipocytes in the upper layer were removed from the filtrate and DMEM/F-12 media containing 10% FBS, 1% penicillin/streptomycin was added to the filtrate to inactivate the collagenase. The digested samples were centrifuged at 1200 *g* for 5 min to obtain the cell pellet, which was then re-suspended in 20 mL erythrocyte buffer (10 min, room temperature) to eliminate the red blood cells. After erythrocyte lysis, the cell suspensions were filtered through a 100 μm nylon mesh before being plated in tissue culture polystyrene flasks. ASCs adhered to the bottom of the flasks within 24 h and growth medium change (low-glucose DMEM containing 10% FBS and 1% penicillin/streptomycin) was performed the second day to remove non-adherent cells. The isolated ASCs were labeled as passage 0 cells and incubated within 37 °C 5% $CO_2$ incubator. Passage 5 ASCs were used for all of the experiments performed in this study.

### 2.2. ASC surface marker characterization by flow cytometry

Passage 5 ASCs were harvested from T75 tissue culture flasks with 0.25% Trypsin-EDTA solution. ASC surface markers CD13, CD29, CD44, CD73, CD90, CD105, CD166, CD14, CD31 and CD45 (Choudhery et al., 2014; Mitchell et al., 2006) were analyzed with flow cytometry. The cells were washed two times with ice-cold PBS before being blocked with Human TruStain FcX™ (1:50 dilution in FACS Buffer (5% FBS, 0.01% Sodium Azide in PBS)) for 20 min at 4 °C. After the block, the cells were centrifuged at 1500 rpm for 5 min at 4 °C and the supernatant were discarded. The cells were then stained with fluorophore-conjugated antibodies: BV650-CD13 (BD Biosciences), PE-CD29 (BioLegend), BB515-CD44 (BD Biosciences), PE Cy7-CD73 (BioLegend), PE Dazzle 594-CD90 (BioLegend), APC-CD105 (BioLegend), BV421-CD166 (BD Biosciences), PE Cy7-CD14 (BioLegend), Alexa Fluor 488-CD31 (BioLegend), APC Cy7-CD45 (BioLegend) at a staining concentration of 1 μg/$10^6$ cells in a total volume of 100 μL FACS buffer (PBS containing 5% FBS and 0.01% Sodium Azide) for 40 min (4 °C, in dark). The cells