

Translation of Adipose-Derived Stem Cells Into Clinical Use to Treat Chronic Wounds

Ren, Guoqiang

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TRANSLATION OF ADIPOSE-DERIVED STEM CELLS INTO CLINICAL USE TO TREAT CHRONIC WOUNDS

**BY
GUOQIANG REN**

DISSERTATION SUBMITTED 2022



AALBORG UNIVERSITY
DENMARK

TRANSLATION OF ADIPOSE-DERIVED STEM CELLS INTO CLINICAL USE TO TREAT CHRONIC WOUNDS

PH.D. DISSERTATION

by

Guoqiang Ren



AALBORG UNIVERSITY
DENMARK

Dissertation submitted 2022

Dissertation submitted: October 2022

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PUBLICATIONS:

1. **Ren G**, Peng Q, Fink T, Zachar V, Porsborg S. Potency Assays for Human Adipose-derived Stem Cells as a Medicinal Product towards Wound Healing. *Stem Cell Research & Therapy* 13, 249 (2022). **IF: 8.1**
2. **Ren G**, Peng Q, Emmersen J, Zachar V, Fink T, Porsborg S. A comparative analysis of wound healing-related heterogeneity of ASCs donors. *Pharmaceutics*. 2022; 14(10):2126. **IF: 6.5**
3. **Ren G**, Juhi M, Peng Q, Fink T, Porsborg S. Selection and validation of reference genes for qPCR of differentiation and maturation of THP-1 cells to M1 macrophage-like cells. *Immunology & Cell Biology*. 2022.02.10. **IF: 5.9**
4. Peng Q, **Ren G**, Xuan Z, Duda M, Pennisi P, Porsborg S, Fink T, Zachar V. Distinct Dominant Lineage from In Vitro Expanded Adipose-Derived Stem Cells (ASCs) Exhibits Enhanced Wound Healing Properties. *Cells*. 2022; 11(7):1236. **(Co-first author)**. **IF: 7.7**
5. Peng Q, Duda M, **Ren G**, Xuan Z, Pennisi P, Porsborg S, Fink T, Zachar V. Multiplex Analysis of Adipose-Derived Stem Cell (ASC) Immunophenotype Adaption to In Vitro Expansion. *Cells*. Jan 2021, Vol 10, No.218. **IF: 7.7**
6. Peng Q, **Ren G**, Zachar V, Porsborg S, Fink T. Evaluation of pentaisomaltose (PIM) as a cryoprotectant for adipose-derived stem cells. **(Co-first author)**. (Manuscript in preparation)
7. **Ren G**, Li B, Li G, Zhang J, Zhu Y, Zou X. Effect of matrix metalloproteinases 9 on the expression of vascular endothelial growth factor in diabetic wound. *Chin J Injury Repair and Wound Healing*. April 2017, Vol 12, No.2.

PREFACE

The purpose of this Ph.D. project was to ease/aid/promote the translation of Adipose-derived stem cells (ASCs) into clinical use to treat chronic wounds.

To achieve this, two primary objectives were investigated:

1. To uncover the ASC-donor variability concerning wound healing properties and point out the source of these differences.
2. To identify how *in vitro* procedures of expansion and storage affect ASC properties.

Supporting this, two hypotheses were proposed:

1. A panel of potential potency assays and markers relevant to wound healing are essential for selecting the best donors and purified ASC subsets for future manufacturing of ASC-based medical products.
2. The *in vitro* procedures of expansion and storage are essential for developing clinically relevant ASC-based medical products.

To address the study's aim and objectives, this dissertation is based on four experimental studies and a review as listed below.

Review: Potency assays for human adipose-derived stem cells as a medicinal product towards wound healing

Guoqiang Ren, Qiuyue Peng, Trine Fink, Vladimir Zachar, Simone R.Porsborg.

Stem Cell Research & Therapy 13, 249 (2022).

Study1: A comparative analysis of wound healing-related heterogeneity of adipose-derived stem cells donors

Guoqiang Ren, Qiuyue Peng, Jeppe Emmersen, Vladimir Zachar, Trine Fink, Simone R. Porsborg

Pharmaceutics 2022; 14(10):2126.

Study2: Selection and validation of reference genes for qPCR of differentiation and maturation of THP-1 cells to M1 macrophage-like cells

Guoqiang Ren, Morten Juhi, Qiuyue Peng, Trine Fink, Simone R. Porsborg

Accepted by Immunology & Cell Biology. 2022.02.10.

Study3: Distinct dominant lineage from in vitro expanded adipose-derived stem cells (ASCs) exhibits enhanced wound healing properties

Qiuyue Peng[†], **Guoqiang Ren**[†], Zongzhe Xuan, Martyna Duda, Cristian P. Pennisi, Simone R. Porsborg, Trine Fink, Vladimir Zachar.

Cells. 2022; 11(7):1236.

Study4: Evaluation of pentaisomaltose (PIM) as a cryoprotectant for adipose-derived stem cells

Qiuyue Peng[†], **Guoqiang Ren**[†], Vladimir Zachar, Simone R. Porsborg, Trine Fink.

Manuscript in preparation

[†] contributed equally.

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My assistant supervisor, Assoc. Prof. Trine Fink, Ph.D., is acknowledged for her co-supervision, beneficial ideas, and constructive discussions. She always opened her door to me and gave her generous and caring support when needed.

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The Laboratory for Regenerative Medicine group, including Assoc. Prof. Cristian Pablo Pennisi, Ph.D., Assoc. Prof. Hiva Alipour, Ph.D., Assoc. Prof. Fereshteh Dardmeh, Ph.D., and technician Lisa Engen are highly acknowledged for their open doors and helping hands.

Lei Liu, M.D., Ph.D., my senior colleague and now the senior director at Novo Nordisk, is highly acknowledged. Without his guidance, I would not be at AAU, and he always selflessly guides me and relieves my confusion when I am helpless.

My dearest office colleague and friend, Ph.D. Qiuyue Peng and Ph.D. Zongzhe Xuan are acknowledged for their timely help and for cheering me up when I fell. It would be utterly different without their company.

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The China Scholarship Council and S.C Van Fonden are acknowledged for their kind financial support during my stay in Denmark.

I would like to extend my deep gratitude to my parents, who always have my back. Over the years, I have been full of guilt toward my parents. I have been studying abroad for many years and cannot be by their side, but they have always supported me spiritually and materially.

Finally, I would like to express my special thanks to my wife, Jianfang, for her dedication and company over the past twelve years and for always supporting me in my studies. I will be your regretless choice in your life. I would also like to thank our beloved daughter, Juni, her birth lit up our lives and brought us endless joy.

ENGLISH SUMMARY

Adipose-derived stem cells are a promising candidate for the treatment of chronic wounds. However, several challenges are to be met before it can be a clinical reality. The fatty tissue from which ASCs are isolated arrives from donors. Nevertheless, ASCs from different donors have been shown to have varying characteristics and potencies. Therefore, a thorough characterization and testing of the potency of the ASCs are essential. A review was conducted to identify potency assays relevant to wound healing, after which the difference between donors was tested, in study 1, in relation to angiogenesis and extracellular matrix (ECM) formation, both central in wound healing. Furthermore, the donor difference regarding the regulation of the inflammation phase of wound healing is crucial, as touched upon in study 2. Finally, the source of these differences was sought in study 3, where distinct subpopulations of ASCs were shown to exhibit enhanced wound healing properties.

When producing advanced therapy medicinal products (ATMPs), as an ASC-based product, several steps in the production can affect the final product. A high number of stem cells are needed for treating a chronic wound, and producing large batches is more cost-effective. Therefore, isolated ASCs are expanded and stored as frozen to provide an off-the-shelf product. To optimize the choice of cryoprotectant used and investigate the equilibration time, study 4 aimed to optimize the freezing procedure.

In study 1, we observed variability in wound healing properties between ASC-donors. When defining the superior ASC-donor based on functional tests relevant to wound healing, we found that angiogenesis and ECM-relevant genes were up-regulated in this. In study 2, our findings demonstrated that the importance of the measured inflammatory response when differentiating the THP-1 monocytes into the M1 phenotype and the anti-inflammatory properties of ASCs might be overlooked when reverse transcription-quantitative polymerase chain reaction (RT-qPCR) data is normalized to inappropriate reference genes. In study 3, we revealed for the first time that the CD274+CD146+ subpopulation of ASCs had increased wound closure capacity and endothelial tube formation potential. In study 4, our results demonstrated that ASCs could be effectively

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cryopreserved in a freezing medium that combined pentaisomaltose (PIM) with a low concentration of Dimethyl sulfoxide (DMSO) and reduced the use of DMSO to only 2.5%, which would be a better choice of CPA for the benefit of ASCs in clinical use.

In conclusion, our findings ensured a better understanding of ASCs and the practical handling of these, to ease the translation of ASCs into clinical use for the treatment of chronic wounds.

DANSK RESUME

Stamceller fra fedtvæv (ASC'er) er en lovende kandidat til behandling af kroniske sår. Flere udfordringer skal dog løses, før det kan blive en klinisk realitet. Fedtvævet, hvorfra ASC'erne isoleres, kommer fra forskellige donorer, som har vist at give anledning til forskellige egenskaber og potent af stamcellerne derfra. Derfor er en grundig karakterisering og test af styrken af ASC'erne afgørende førend de tages i brug. En gennemgang af den eksisterende litteratur blev udført for at identificere potensassays relevante for sårheling og som også kan kvantificerer forskellen mellem donorer. Nogen af disse blev afprøvet i Study 1, hvor ASC'er blev testet i relation til angiogenese og ECM-dannelse, begge centrale processer i sårheling. Ydermere er forskellen mellem donorer med hensyn til reguleringen af inflammationsfasen afgørende, som berørt i Study 2. Endelig blev kilden til oprindelsen af disse forskelle søgt i Study 3, hvor forskellige subpopulationer af ASC'er viste sig at have forskellige sårhelende egenskaber.

Ved fremstilling af lægemidler til avanceret terapi (ATMP'er), som et ASC-baseret produkt er, kan flere trin i produktionen påvirke slutproduktet. Et stort antal stamceller er nødvendige for at behandle et kronisk sår, og det er mere omkostningseffektivt at producere større partier. Derfor ekspanderes isolerede ASC'er og opbevares derefter på frost, for at have et produkt klar når behovet melder sig. For at kunne fryse stamcellerne uden at de tager skade deraf, benyttes et kryobeskyttelsesmiddel. For at optimere valget af kryobeskyttelsesmiddel, sigtede Study 4 på at finde en bedre løsning ind den eksisterende.

I Study 1 observerede vi variation i sårhelingsegenskaber mellem ASC'er fra forskellige donorer. I jagten på den overlegne ASC-donor identificeret ud fra funktionelle test for sårheling, fandt vi, at angiogenese- og ECM-relevante gener var opreguleret i de mest potente ASC'er. I Study 2 viste vores resultater, at det målte inflammatoriske respons ved differentiering af THP-1-monocyterne til M1-fænotypen og de antiinflammatoriske egenskaber af ASC'er kan være fejlagtigt undervurderede, når omvendt transkription-kvantitativ polymerasekædereaktion (RT-qPCR)-analyser normaliseres med ustabile referencegener. I Study 3 afslørede vi for første gang, at den CD274+CD146+ subpopulation af ASC'er er

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overlegne i at forbedre sårhelingspotentialet og kjardannelsen. I Study 4 viste vores resultater, at ASC'er effektivt kunne kryokonserveres i et frysemedium, der kombinerede pentaisomaltose (PIM) med en lav koncentration af Dimethylsulfoxid (DMSO) og reducerede brugen af DMSO til kun 2,5 %, hvilket kunne fordele for den kliniske brug af ASC'er.

Som konklusion fremmer vores resultater en bedre forståelse af ASC'er og den praktiske håndtering af disse, alt sammen for at lette ibrugtagningen af ASC'er til klinisk behandling af kroniske sår.

ABBREVIATIONS

ACTB: β -actin

ASCs: Adipose-derived stem cells

ATMPs: Advanced therapy medicinal products

B2M: β 2-microbulin

BMI: Body mass index

BMSCs: Bone marrow mesenchymal stem cells

CCL2: C-C Motif Chemokine Ligand 2

CFU-F: Colony-forming unit-fibroblastic

CM: Conditioned medium

CPA: Cryoprotectant agent

DMEM: Dulbecco's minimum essential medium

DMSO: Dimethyl sulfoxide

ECM: Extracellular matrix

EGF: Epidermal Growth Factor

ELISA: Enzyme-linked immunosorbent assay

EMA: European Medicines Agency

EVs: Extracellular vehicles

FBS: Fetal bovine serum

FGF: Fibroblast growth factor

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GMP: Good manufacturing practice

GOI: Genes of interest

HGF: Hepatocyte growth factor

HPL: Human platelet lysate

IDO: Indoleamine 2,3-dioxygenase

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IFATS: International Federation for Adipose Therapeutics and Science

IFN- γ : Interferon- γ

IGF-1: Insulin-like growth factor-1

IL: Interleukin

ISCT: International Society for Cellular Therapy

KGF: keratinocyte growth factor

LPS: lipopolysaccharide

MeA: Mechanisms of Action

MMP: Matrix metalloproteinases

MoA: Modes of Action

MSCs: Mesenchymal Stem Cells

PDGF: Platelet-derived growth factor

PIM: Pentaismaltose

PMA: Phorbol 12-myristate 13-acetate

PPIA: Peptidylprolyl Isomerase A

RPL37a: Ribosomal protein L37a

RT-qPCR: Reverse transcription quantitative polymerase chain reaction

SVF: Stromal vascular fraction

TGF- β : Transforming growth factor-beta

TIMP1: TIMP metalloproteinase inhibitor 1

TNFA: Tumor necrosis factor-alpha

UBC: Ubiquitin C

VEGF: Vascular endothelial growth factor

α -MEM: Alpha minimum essential medium

CHAPTER 1. INTRODUCTION

1.1 WOUND HEALING

Skin, the largest organ in the body, protects us as a barrier against harmful environmental factors, and its damage must be mended efficiently and adequately [1]. Regular wound healing is characterized by a multi-step interactive process that causes restoring a functional dermis/epidermis layer [2]. This procedure can be split into three distinctive programmed stages: 1) hemostasis/inflammation, 2) proliferation, and 3) remodeling [3].

These stages and their physiological functions need to occur in the appropriate order, at a given time, with the aid of all skin cells such as endothelial cells, fibroblasts, keratinocytes, immune cells, and blood cells, i.e., leukocytes, erythrocytes, thrombocytes [4].

The initial healing stage is characterized by a hemostasis/inflammation reaction to control bleeding at the wound site via an intricate communication of activated cells with coagulation proteins and complement mediators [3]. In the inflammation phase, neutrophils and macrophages infiltrate the wound area quickly and produce cytokines to augment the healing process. This process leads to granulation tissue formation, favoring the transition from inflammation to repair [2].

The proliferation stage is characterized by angiogenesis and re-epithelialization and massive proliferation and migration of fibroblasts, which are required to secrete the amounts of extracellular matrix (ECM) necessary to replace the lost tissue. Basal epithelial cells divide, and the keratinocytes begin to migrate as sheets from the wound edging [5].

When it comes to the remodeling stage, reestablishing a regular blood distribution affords a healthy microcirculation environment for the migration and proliferation of epidermal and dermal cells. In turn, this gives rise to wound re-epithelialization and rebuilding of epidermal integrity. The proliferation and ECM synthesis of fibroblasts in the wound contribute to forming the granulation tissue suffused with afresh-

formed blood vessels. Next, wound contraction and matrix remodeling occur, and the damage becomes firm and relatively avascular, giving rise to highly collagenized scar tissue [6,7].

All the above phases must function properly and at specific times for a routine healing process. Thus, an impediment to any of these three stages of wound healing may impair the healing process, and the wound will become chronic [7].

1.2 CHRONIC WOUNDS

Chronic wounds are described as non-healing wounds or ulcers that are difficult to heal in six weeks and are of very different etiology. These wounds affect millions of people worldwide, and 1 - 2% of the population will sustain a chronic wound during their lifetime [8]. A chronic wound reduces the quality of life for the patient owing to substantial functional loss, psychosocial morbidity, and an increased risk of limb amputation [9]. Also, for society, the economic costs of wound therapy are appraised to constitute about 2 - 4% of the total healthcare budget inside the EU [10].

The major types of chronic wounds are venous ulcers, diabetic ulcers, and pressure ulcers [11]. Venous ulcers commonly result from hypoxia in the legs of venous congestion and are instigated by blood circulation problems caused by obstructed veins or malfunctioning blood valves [12]. Diabetic ulcers are often a result of neuropathic impairment, immune dysfunction, and peripheral vascular disease, which lead to these wounds becoming severely infected [13]. Pressure ulcers primarily occur in paralyzed or unconscious patients bedridden or with limited mobility. These ulcers stem from ischemia owing to persistent pressure over a bony protruding [14].

The underlying mechanism of chronic wound development is complex and involves a multifactorial etiology, including prolonged infections, the formation of drug-resistant bacteria and biofilms, persistent aberrant inflammation, dysfunctional fibroblasts and basal epithelial cells failing to respond to repair stimuli, reduced angiogenesis, defective recruitment and activation of stem cells, increased level of inflammatory cytokines, metalloproteases, and reactive oxygen species [7,15]. In

general, these pathophysiologic phenomena fail these wounds to heal (Fig.1).

In most chronic wounds, the healing process is engaged in the inflammatory stage, leading to pathological inflammation and the failing endogenous repair reaction to getting into the improved phases of wound healing. The permanent inflammatory stage produces abnormal inflammatory cytokines [16]. Meanwhile, the growth in inflammatory cells results in degradation of ECM caused by an augment in the secretion of matrix metalloproteinases (MMPs) and loss of valuable wound healing growth factors, such as transforming growth factor-beta (TGF- β), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and hepatocyte growth factor (HGF) [17,18]. Reducing the factors released by local inflammatory cells and decreasing vascularization are significant obstacles to healing.

Typically, the dermal layer wounds or its partly extended wounds are able to regenerate. However, deep skin wounds are hard to heal effectively by the body. As the limited sources of cells for regeneration, excluding the wound edges, completed recovery and re-epithelialization of deep skin wounds often take a long time. These wounds are difficult to heal fully, usually accompanied by contractures and scar formation, and are short of completely restoring the dermis [19].

Thus, the management strategy for treating chronic wounds is to return them to the normal progression of wound healing. Current treatments are based on an element of wound bed preparation, infection treatment, maintaining an adequately moist environment, and surgical repair in severe cases. Nonetheless, a considerable proportion of patients remain non-responsive to these treatments, underscoring the need to develop more efficient therapies.

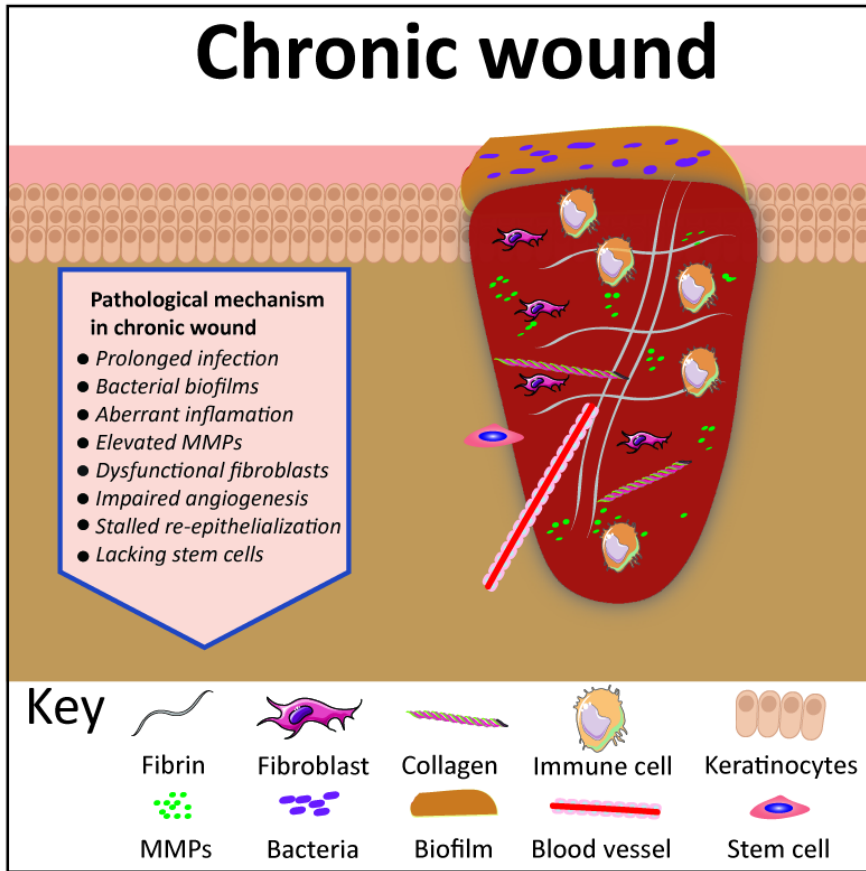


Figure 1. The pathological mechanism in a chronic wound. MMPs: Matrix metalloproteinases.

1.3 ADIPOSE-DERIVED STEM CELLS

In recent years, cell-based therapies are gradually becoming a reality in routine medical management and, mainly, in skin wound treatment as they are able to repair/replace injured tissue with a natural one owing to their innate capability to yield beneficial factors essential for wound healing. They offer promises to repair and/or replace injured tissue and restore damaged function [20].

Stem cells have emerged as a novel cell-based therapy for repairing damaged tissue based on stimulating the body's repair mechanism to regenerate tissues and restore normal function. Mesenchymal Stem

Cells (MSCs) are multipotent adult stem cells that are able to self-renew, be proliferative and differentiate into multiple cell lineages, including adipocytes, osteocytes, chondrocytes, myocytes, and tenocytes [21]. The cell surface markers are often used to distinguish MSCs, and they are generally positive for CD44, CD73, CD90, CD105, and Stro-1 and negative for CD14, CD34, CD45, and HLA-DR [21].

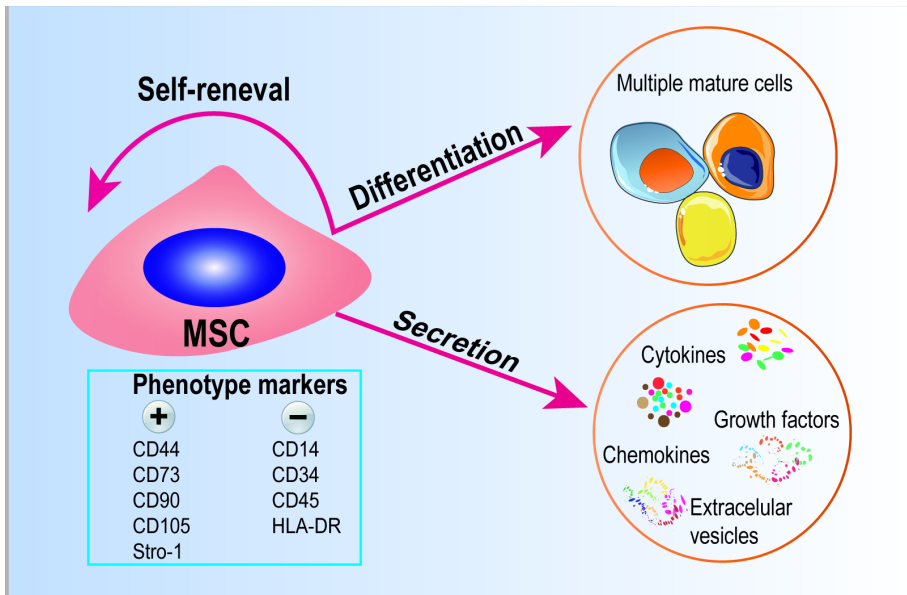


Figure 2. Characterization of MSC. MSCs can self-renew, proliferate, differentiate into multiple cell lineages, and produce cytokines and molecules to play their biological role. MSCs are often distinguished by a series of cell surface markers. MSCs: Mesenchymal Stem Cells.

MSCs are most regularly isolated from bone marrow or adipose tissue, termed bone marrow mesenchymal stem cells (BMSCs) or adipose-derived stem cells (ASCs) [22]. Comparing ASCs to BMSCs shows comparable morphology, cell surface markers, and differentiation potential. The transcriptomic and proteomic study also displays a similar profile for ASCs and BMSCs [23,24]. The advantages of ASCs over BMSCs and other stem cell types are that they are reasonably straightforward to acquire in their thousands from basic liposuctions and provide a much higher yield [24,25]. Furthermore, ASCs can maintain their characteristics after long-standing in vitro culture and retain low immunogenicity, enabling the use of ASCs from different donors [26,27].

To date, most preclinical studies are based on BM-MSCs. Nevertheless, ASCs and BM-MSCs share many biological properties, so considerable knowledge concerning BM-MSCs can be straightforwardly utilized by the ASCs [25]. Based on these advantages, ASCs are being investigated as a therapeutic strategy for various pathological conditions, including hard-to-heal wounds.

1.4 ASCS IN WOUND HEALING

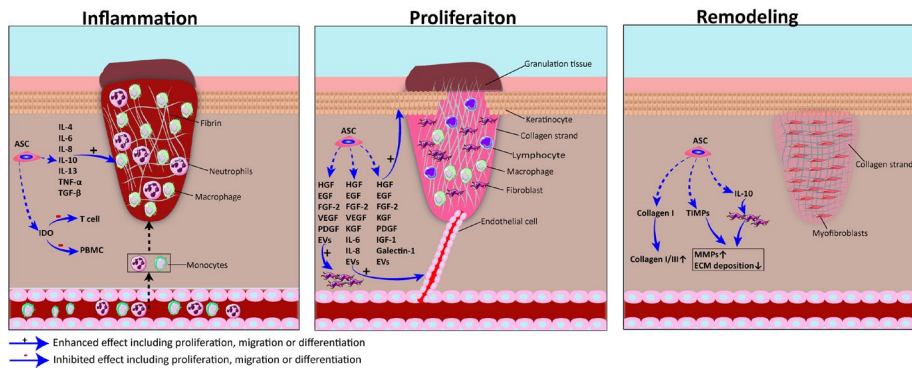


Figure 3. Underlying Modes and Mechanisms of action for ASCs in different phases of wound healing. ASCs have a distinct MoA in each phase of wound healing, but the MeA by which and how to achieve these are still not entirely understood. During the Inflammation phase, ASCs play the immunomodulatory effect mainly by secretion of bioactive factors. In the proliferation phase, ASCs stimulate angiogenesis and support fibroblast and keratinocyte growth by secretion of bioactive factors. Relevant to the remodeling phase, ASCs participate in the ECM's composition, deposition, and degradation mainly by secreted type I collagen, IL10, and TIMP1. ASC: Adipose-derived stem cell; PBMCs: Human peripheral blood mononuclear cells; IDO: Indoleamine 2,3-dioxygenase; IL: Interleukin; TGF- β : Transforming growth factor-beta; TNF- α : Tumor necrosis factor-alpha; HGF: Hepatocyte growth factor; EGF: Epidermal Growth Factor; FGF: Fibroblast growth factor; VEGF: Vascular endothelial growth factor; PDGF: Platelet-derived growth factor; EVs: Extracellular vesicles; KGF: keratinocyte growth factor; IGF-1: Insulin-like growth factor-1; MoA: Modes of Action; MeA: Mechanisms of Action; ECM: Extracellular matrix; MMP: Matrix metalloproteinases; TIMP1: TIMP metalloproteinase inhibitor 1.

Currently, ASCs are employed in many early clinical trials for inflammatory and autoimmune diseases, ischemic heart disease, limb ischemia, fistulas, liver disease, and orthopedics, where their safety and efficiency have been verified and revealed great promise [28–35]. Cutaneous wounds treated with ASCs have exhibited great therapeutic potential [36–38]. Even though the mechanism of action by how ASCs hasten

wound healing is not fully understood. It has been conjectured that ASCs employ their wound-healing properties by lessening inflammation, stimulating angiogenesis, and supporting fibroblast and keratinocyte growth [39].

Abundant *in vitro* and *in vivo* studies have struggled to clarify the role of ASCs in the different stages of wound healing [40–43].

During the inflammation phase, ASCs have been found to decrease the pro-inflammatory response and accelerate the switch from the inflammatory to an anti-inflammatory phase [43–47]. ASCs play the immunomodulatory effect mainly with the aid of the secretion of bioactive factors, i.e., indoleamine 2,3-dioxygenase (IDO), interleukin 4/6/8/10/13 (IL-4/6/8/10/13), tumor necrosis factor-alpha (TNF- α), HGF, and TGF- β , but the effect of direct cell-cell contact and exosomes and has also been indicated [40,44,48–57]. These effects have been additionally confirmed by *in vivo* studies [50,58,59].

Relevant to the proliferation phase, ASCs play a positive role in the formation of granulation by the secretion of growth factors, i.e., FGF-2, epidermal growth factor (EGF), VEGF, HGF, and PDGF-AA, and extracellular vesicles (EVs) to promote fibroblast proliferation and migration and the production and modulation of ECM by fibroblasts, i.e., Secretion of Collagen1, Collagen11, Elastin, Fibronetic, matrix metalloproteinase-1/2/9 (MMP-1/2/9) and TIMP metalloproteinase inhibitor 1 (TIMP1) [60–66]. Furthermore, ASCs have demonstrated to enhance the process of angiogenesis by secreting the angiogenic growth factors, i.e., VEGF, EGF, HGF, FGF-2, keratinocyte growth factor (KGF), IL-6, and IL8, and EVs to encourage endothelial cell proliferation and migration and differentiate into endothelial cells that contribute to increased neovascularization [62,66–73]. Moreover, ASCs have been shown to accelerate the re-epithelization by promoting keratinocyte proliferation and migration through paracrine secretion of various paracrine factors, i.e., Galectin-1, FGF-2, EGF, HGF, KGF, insulin-like growth factor-1 (IGF-1), and PDGF, and EVs [60,62,66,74–76].

During the remodeling phase, the evidence of the effect of ASCs often hard to distinctly differentiate between this phase and the granulation tissue formation stage since it may not be straightforward to distinguish

the formation of granulation tissue and its remodeling utilizing in vitro assay. Still, ASCs have shown the capacity to participate in the composition, deposition, and degradation of ECM, either by regulating their own ECM production by secreted type1 collagen, IL10, and TIMP1 or ECM modulation generated by fibroblasts [63,77–80].

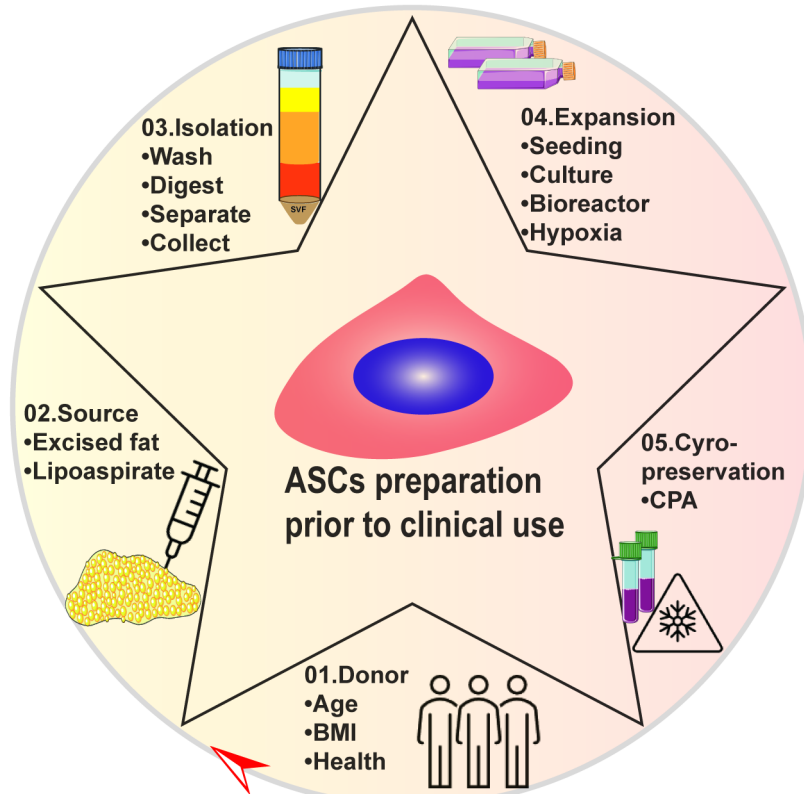


Figure 4. Critical steps in the preparation of ASCs prior to clinical use. A series of steps, including the donor's choice, the source of subcutaneous fat, the isolation and expansion steps, and the CPA selection for cryopreservation of ASCs, are essential in the preparation of ASCs before being applied to clinical use. ASCs: Adipose-derived stem cells; BMI: Body mass index; CPA: Cryoprotectant agent.

1.5 ASCS IN CLINICAL USE

When promoting the translation of ASCs from pre-clinical studies into clinical practice, several vital steps in harvest, isolation, expansion, and

issues concerning characterization, potency testing, and cryopreservation need to be thoroughly assessed.

1.5.1 HARVEST OF ADIPOSE TISSUE

The selection of donors from which to harvest adipose tissue is the first step toward producing an ASCs-based product.

Quite a few studies have identified the variability in ASCs isolated from different donors. The majority of studies were focused on age [81–86], and body mass index (BMI) [87,88] and higher age or weight of donors may negatively affect the viability [82], proliferation [81,82,84], migration [85], angiogenic [86,87], and differentiation [81–83,88] capacity of the ASCs. A recent study observed that ASCs from old donors have a lower ability to promote fibroblast migration and endothelial cell angiogenesis than those from young donors [85]. In addition to the effect of age and BMI, the physical condition of the donor also affects the properties of ASCs. It is worth noting that ASCs isolated from diabetic patients were shown to have inferior capabilities of proliferation, migration, and pro-angiogenesis [86,89,90]. However, no study has reported which donors are most suitable for specific therapy applications such as wound healing. Thus, developing a panel of markers by which to identify the best donor to isolate ASC from and to ensure the best treatment effect is essential.

Subcutaneous fat is an ideal source of ASCs proper for clinical use as they can be accessed with ease and yield better than other anatomical regions [91–93]. Thereinto, tissue resection, and liposuction are the primary approaches to isolating ASCs. These two methods have been found comparable in terms of cell yield [94]. Due to the invasiveness and complexity of practice for surgical tissue resection, liposuction seems to be a better approach to obtaining adipose tissue and can easily be achieved from cosmetic/aesthetic surgery.

1.5.2 ISOLATION AND EXPANSION OF THE ASCS

ISOLATION

The next step after the collected adipose tissue is isolating ASCs from the stromal vascular fraction (SVF). SVF is a diverse collection of cells enclosed in adipose tissue, and their cellular components have been illustrated in Fig.5. Firstly, the fatty tissue must be washed to eliminate most of the erythrocytes, digested with proteases, centrifuged to remove tissue remnants and mature adipocytes, lysed to remove the residual erythrocytes, and finally collected in a single-cell suspension for seeding to further select ASCs. Based on the adhesive capability of ASCs, the cells are isolated and defined as pure since other adherent non-proliferative cells and remaining floating cells will be removed after a few passages.

Zuk et al. advocated the principle for the isolation of ASCs for the first time in 2001 [95]. However, over the years, different laboratories set their standards and desired practices with an army of modifications based on this, and no novel isolation protocol has been established to provide a considerably better cell yield than the original. Thus, the criteria for good manufacturing practice (GMP) are urgently set.

EXPANSION

To achieve a sufficient number of ASCs for clinical purposes, the ASCs typically need to be expanded. For the culture of ASCs, a standard culture medium combination, the alpha minimum essential medium (α -MEM) or Dulbecco's minimum essential medium (DMEM) complete with 10% fetal bovine serum (FBS) at a physiological glucose concentration (100 mg/dl; 5.6 mm) has been proposed and extensively used [96]. It is worth noting that ASCs cultured in α -MEM have been proved to proliferate faster than in DMEM, thus making the α -MEM a preferred choice [97].

Although FBS has been widely used for culturing ASCs in pre-clinical studies, there is a significant barrier to clinical translation due to the inconsistency and immunogenicity issues of FBS [98]. To avoid this issue, an alternative to FBS could be human serum-derived products,

such as human platelet lysate (HPL), which can significantly accelerate ASC proliferation compared to FBS [97]. In addition, using HPL to expand superior ASCs has been confirmed to ensure a similar proliferation or differentiation potential as FBS [99]. However, more work is needed to evaluate the efficiency and reliability of the ASC culture medium compositions before clinical use.

Oxygen tension is another factor in optimizing the ASCs expansion. Regularly, the cultivation of ASCs proceeds in an atmosphere of 20% oxygen tension; however, it has been established that hypoxic preconditioning ASCs by brief exposure to hypoxia in 1-5% oxygen concentration has a favorable effect on the ASC proliferation, viability, and stemness maintenance [100–105]. It has been found that by exposing the ASCs to hypoxic conditions, several of the wound-healing properties are affected, including enhanced immunosuppression [106], secretion of pro-angiogenesis growth factors [105,107–111], increased endothelial cell growth [107] and angiogenesis [105,109–111], re-epithelialization [90,112], and ECM production [113]. In addition, a positive effect of hypoxic preconditioning on ASCs from a distinct donor group is that hypoxia can switch the low angiogenic function in the cells of old or diabetic individuals [86,89]. In light of the benefits of hypoxic preconditioning of ASCs, it can be predicted that it will be a regular part of GMP practices on ASC expansion.

Bioreactors play a significant role in the large-scale production of ASCs. To date, the stirred-tank and the hollow-fiber bioreactors are the most frequently used in the cell-based product and have shown great promise to be broadly used in the GMP-compliant expansion of MSCs for clinical applications [114–116]. Nevertheless, preclinical data for the expansion of ASCs collected from the automatic bioreactor are few, and carefully evaluating the role of the bioreactor to ensure quality control in a standardized manner is necessary.

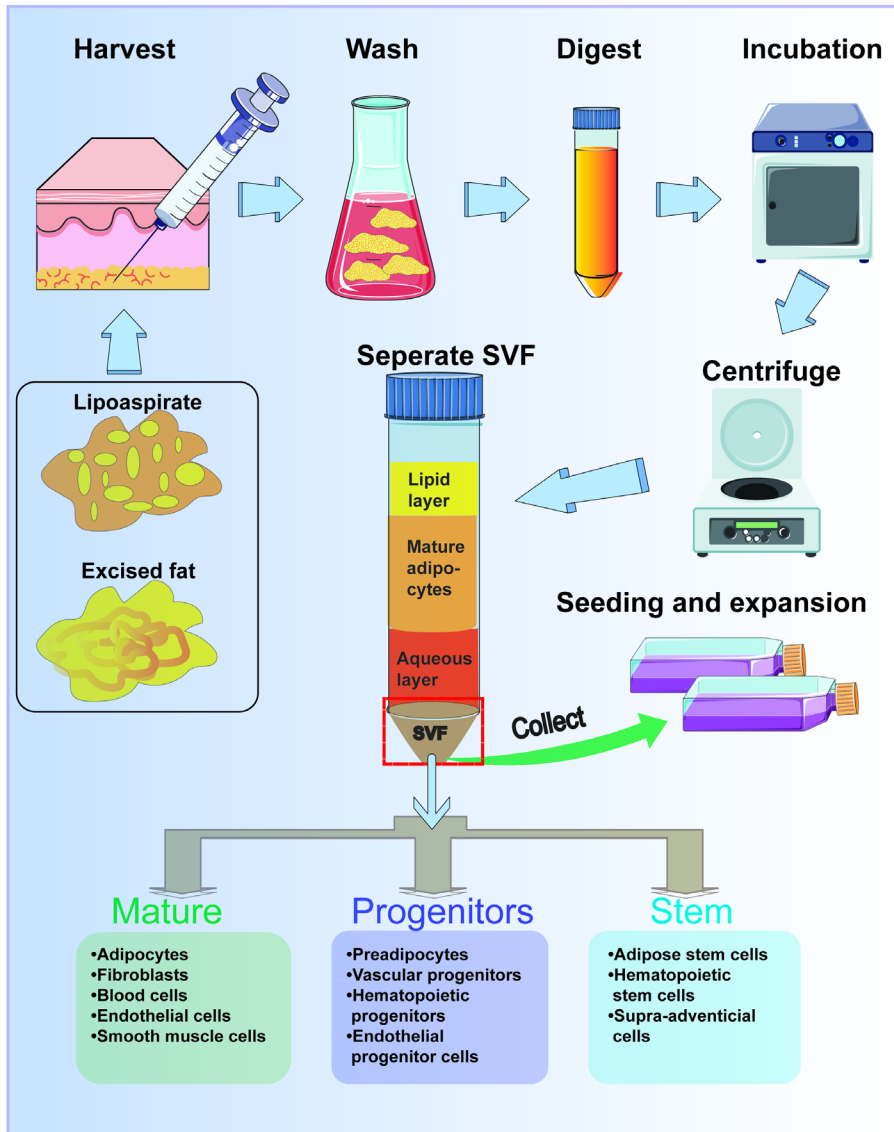


Figure 5. Overview of the isolation process for ASCs. Lipoaspirates were washed, digested, centrifugated, and separated into a layer of high-fat droplets from mature adipocytes. The SVF was seeded and expanded in the culturing flasks after removing all the layers above SVF. ASCs: Adipose-derived stem cells; SVF: Stromal vascular fraction.

1.5.3 ASCS ISSUES PRIOR TO CLINICAL USE

CHARACTERIZATION

The European Medicines Agency (EMA) has proposed that stem cell-based medicinal products before clinical use are tested to identify stem cells, and the tests should quantify the self-renewal ability and the expression of specific surface markers [117]. A batch of guidelines for the minimal criteria of ASCs has been further recommended by the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT) [118]. These criteria include the ability for self-renewal tested by colony-forming unit-fibroblastic (CFU-F) assay, viability tested by either flow cytometry or microscopic inspection, the trilineage differentiation potential tested by histochemistry, enzyme-linked immunosorbent assay (ELISA), reverse transcription-quantitative polymerase chain reaction (RT-qPCR), or Western blot, and the expression of the following specific surface markers: positive for CD13, CD29, CD44, CD73, CD90, CD105, and negative for CD31, CD45, CD235a. However, these criteria are only for identifying ASCs, not for testing the specific potency towards wound healing, which are probably more valuable when ASCs are applied clinically.

POTENCY TESTING FOR WOUND HEALING

The EMA suggests that a potency assay for stem cell-based products should involve the quantitative measure of the biological or cellular mechanism of action. On top of this, the potency assay should include the quantitative measure of product characteristics and/or functional activity associated with the Mechanisms (MeA) and/or modes (MoA) of Action of the cell-based medicinal products [119,120]. The MeA is the potential molecular basis regulated by ASCs, such as “up-regulation of the *VEGF* gene”, and the MoA is the functional activity induced by ASCs, such as “pro-angiogenesis ability” [121]. To better evaluate the potential role of ASCs in the wound healing process, potency assessments are divided into two types of assays: surrogate assays and biological assays. The purpose of surrogate assays is to measure the product parameters linked to ASC MeA, including surface markers, gene transcription, and soluble mediators detected in ASC. The biological

assays assess the product activity correlated with its MoA at a certain dose in a related biological system and frequently include in vivo models, in vitro culture systems, or a combination [121,122]. Typically, a single surrogate or biological assay might be inadequate for a comprehensive assessment of potency; thus, a combination of the surrogate assay, surrogate and biological assays, or biological assays alone are needed to validate the stableness and reliability of the ASC product [122]. Ren et al. have provided a detailed overview of the potency assays for ASCs as a medical product to wound healing in the review [121].

For potency assays of ASCs, RT-qPCR has been widely applied to evaluate the expression of gene transcription in ASCs or their target cell. As a potent tool, RT-qPCR is a fast and sensitive procedure for measuring low abundance mRNA expression and determining differences in expression levels by scaling raw expression data from genes of interest (GOI) to a stably expressed reference gene [123]. However, a reliable reference gene is strictly required to assess the results obtained correctly, as RT-qPCR data depends upon many factors, including RNA quality and quantity and reverse transcriptase efficiency [124,125]. Therefore, it is essential to normalize the expression of a GOI using the reference gene(s), whose expression level is not regulated by the specific experimental conditions for all samples, as an internal control [126]. The published MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines recommended validating the reference gene for each set of RT-qPCR experiment conditions [126]. Indeed, once the most stable gene(s) has been rigorously substantiated, it may be used as a criterion for the specific experimental approach, allowing better reproducibility results for other researchers. Therefore, choosing and evaluating the potential reference genes must be thoughtful when elucidating biological responses for performing potency assays of ASCs.

CRYOPRESERVATION

The safe and efficient cryopreservation of ASCs products is an essential procedure after isolation and expansion and ensures the viability and function of the ASCs before clinical use. As a universally used gold standard cryoprotectant agent (CPA), DMSO prevents the formation of

intracellular and extracellular crystals; otherwise, the cells would be lysed by ice crystals and dead after thawing. Though, DMSO exerts toxic side effects on cells, primarily relying upon the DMSO concentration and cell exposure time throughout the pre-freezing and post-thawing phases [127–129]. The most commonly used DMSO concentration is 10%, and as DMSO has concentration-dependent cell toxicity; thus, lowering the DMSO concentration may be an ideal option. However, a study found that reducing the DMSO concentration seems to contribute to decreasing MSCs survival and recovery [130]. Other CPAs have been considered to replace or reduce DMSO for the cryopreservation of MSCs. PIM, a potential new CPA, has been approved for clinical use. A recent study found that ASCs could be effectively cryopreserved in a freezing medium that contained 10% PIM and only 2% DMSO, at which post-thaw recovery was still maintained and the toxic effects of DMSO reduced [131]. Still, more studies are required to explore the cryoprotective effect of PIM as a novel CPA for ASCs.

GUOQIANG REN

TRANSLATION OF ADIPOSE-DERIVED STEM CELLS INTO CLINICAL USE TO TREAT CHRONIC WOUNDS

CHAPTER 2. OBJECTIVES

To ease/aid/promote the translation of Adipose-derived stem cells (ASCs) into clinical use to treat chronic wounds, two primary objectives were investigated:

1. To uncover the ASC-donor variability concerning wound healing properties and point out the source of these differences.
2. To identify how *in vitro* procedures of expansion and storage affect ASC properties.

Supporting this, two hypotheses were proposed:

1. A panel of potential potency assays and markers relevant to wound healing are essential for selecting the best donors and purified ASC subsets for future manufacturing of ASC-based medical products.
2. The *in vitro* procedures of expansion and storage are essential for developing clinically relevant ASC-based medical products.

To answer the hypothesis as described, a review and four experimental studies were carried out:

Review: To facilitate the advancement of future potency assays within the area of chronic wounds.

Study 1: To control the product consistency of stem cells when developing ATMPs, we aim to identify a panel of biomarkers, especially related to wound healing, to be used in the future screening of ASCs for downstream production.

Study 2: To select and validate the most stable reference genes for qPCR when assessing the differentiation of THP-1 monocytes into macrophage-like cells and M1 phenotype.

Study 3: To compare the functionality of distinct ASC-subpopulations in terms of wound healing properties.

GUOQIANG REN

TRANSLATION OF ADIPOSE-DERIVED STEM CELLS INTO CLINICAL USE TO TREAT CHRONIC WOUNDS

Study 4: This study aimed to optimize the freezing procedure for ASC-based therapies by comparing the cryoprotective effect of different concentrations of DMSO and pentaisomaltose (PIM) as a novel CPA.

CHAPTER 3. METHODS AND RESULTS

Detailed methods and results from all studies in this thesis are presented in manuscripts 1-5, respectively (see appendices). An overview of the main methods and findings is presented below.

3.1 REVIEW

Review | [Open Access](#) | [Published: 11 June 2022](#)

Potency assays for human adipose-derived stem cells as a medicinal product toward wound healing

[Guoqiang Ren](#), [Qiyue Peng](#), [Trine Fink](#), [Vladimir Zachar](#) & [Simone Riis Porsborg](#) 

[Stem Cell Research & Therapy](#) **13**, Article number: 249 (2022) | [Cite this article](#)

1134 Accesses | 2 Altmetric | [Metrics](#)

Abstract

In pre-clinical studies, human adipose-derived stem cells (hASCs) have shown great promise as a treatment modality for healing of cutaneous wounds. The advantages of hASCs are that they are relatively easy to obtain in large numbers from basic liposuctions, they maintain their characteristics after long-term in vitro culture, and they possess low immunogenicity, which enables the use of hASCs from random donors. It has been hypothesized that hASCs exert their wound healing properties by reducing inflammation, inducing angiogenesis, and promoting fibroblast and keratinocyte growth. Due to the inherent variability associated with the donor-dependent nature of ASC-based products, it appears necessary that the quality of the different products is prospectively certified using a set of most relevant potency assays. In this review, we present an overview of the available methodologies to assess the Mode and the Mechanism of Action of hASCs, specifically in the wound healing scenario. In conclusion, we propose a panel of potential potency assays to include in the future production of ASC-based medicinal products.

Method:

Based on a thorough literature review, we critically collected the current evidence on the subject of the hASC MoA and MeA.

Results:

GUOQIANG REN

TRANSLATION OF ADIPOSE-DERIVED STEM CELLS INTO CLINICAL USE TO TREAT CHRONIC WOUNDS

We proposed a panel of potency assays to be included in the production of future ASC-based medicinal products, particularly in the wound healing setting.

3.2 STUDY 1



Article

A Comparative Analysis of the Wound Healing-Related Heterogeneity of Adipose-Derived Stem Cells Donors

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Abstract: Adipose-derived Stem cells (ASCs) are on the verge of being available for large clinical trials in wound healing. However, for developing advanced therapy medicinal products (ATMPs), potency assays mimicking the mode of action are required to control the product consistency of the cells. Thus, greater effort should go into the design of product assays. Therefore, we analyzed three ASC-based ATMPs from three different donors with respect to their surface markers, tri-lineage differentiation, proliferation, colony-forming unit capacity, and effect on fibroblast proliferation and migration, endothelial proliferation, migration, and angiogenesis. Furthermore, the transcriptome of all three cell products was analyzed through RNA-sequencing. Even though all products met the criteria by the International Society for Cell and Gene Therapy and the International Federation for Adipose Therapeutics and Science, we found one product to be consistently superior to others when exploring their potency in the wound healing specific assays. Our results indicate that certain regulatory genes associated with extracellular matrix and angiogenesis could be used as markers of a superior ASC donor from which to use ASCs to treat chronic wounds. Having a panel of assays capable of predicting the potency of the product would ensure the patient receives the most potent product for a specific indication, which is paramount for successful patient treatment and acceptance from the healthcare system.

Keywords: adipose-derived stem cells; heterogeneity; transcriptome; wound healing; angiogenesis; extracellular matrix

Citation: Ren, G.; Peng, Q.; Emmersen, J.; Zachar, V.; Fink, T.; Porsborg, S.R. A Comparative Analysis of the Wound Healing-Related Heterogeneity of Adipose-Derived Stem Cells Donors. *2022*, *14*, 2126. <https://doi.org/10.3390/pharmaceutics14102126>

Method:

We comprehensively characterize three ASC-based ATMPs from three different donors and their inter-donor distinctions conditional on a group of quantitative characteristics and functional parameters. The parameters were surface markers, tri-lineage differentiation, proliferation, colony-forming unit capacity, and effect on fibroblast proliferation and migration, endothelial proliferation, migration, and angiogenesis, representing various wound healing processes. Besides, the transcriptome of all three ASC-based ATMPs was examined by RNA sequencing.

Results:

- 1) The histochemical assessment demonstrated that all cultures have the ability to differentiate into adipocytes, osteocytes, and chondrocytes (see appendices manuscript 2, Fig.2A).
- 2) The colony-forming potential was superior for ASC-106 and ASC-105 compared to ASC-101 (n=5 represents biological replicates, see appendices manuscript 2, Fig.2B).
- 3) The proliferative capacity of ASC-106 and ASC-105 was equivalent and both notably faster than that of ASC-101 (n=3 represents biological replicates, see appendices manuscript 2, Fig.2C).
- 4) All three cultures were positive for the characteristic markers CD105, CD73, CD90, and our selected ASC-markers CD146 and CD34. Besides, a deficient expression of CD31 was noticed (see appendices manuscript 2, Fig.2D).
- 5) The quantifiable assessment of the scratch closure showed CM obtained from ASC-106 and -105 to improve the migration of fibroblasts and endothelial cells to a higher level than that obtained from ASC-101 (n=8 represents technical replicates, see appendices manuscript 2, Fig.3B and 4B).
- 6) CM from ASC-106 and -105 considerably outperformed that of ASC-101 when calculating fibroblasts and endothelial cell proliferation (n=3 represents biological replicates, see appendices manuscript 2, Fig.3C and 4C).
- 7) The tube formation assay was utilized to assess angiogenic ability. It was observed that CM obtained from ASC-106 and ASC-105 was superior to that from ASC-101 in all assessed parameters, including branching length, extreme number, node number, and junction number (n=5 represents biological replicates, see appendices manuscript 2, Fig.4E).
- 8) RNA sequencing was used to investigate the transcriptomic differences between ASC-donors. From the pool of 24237 genes, 158 genes were upregulated in both ASC-105 and -106 compared to ASC-101. When defining the ASC-106 and -105 donor as superior based on functional tests relevant to wound healing, we identified 35 up-regulated genes, especially related to angiogenesis and ECM-relevant genes (see appendices manuscript 2, Fig.5 and 6, and Table 2 and S1).

3.3 STUDY 2

► Immunol Cell Biol. 2022 Oct 2. doi: 10.1111/imcb.12590. Online ahead of print.

Selection and validation of reference genes for qPCR of differentiation and maturation of THP-1 cells to M1 macrophage-like cells

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Affiliations + expand

PMID: 36184577 DOI: 10.1111/imcb.12590

Abstract

For cell-based assays studying monocytes and macrophages, the immortalized monocyte cell line THP-1 is widely used and stimulated with phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), and/or interferon- γ (IFN- γ) it differentiates and polarizes into pro-inflammatory M1-like macrophages. For the quantification of this and the effect of different factors affecting these processes, the expression levels of various maturation markers are determined using reverse transcription-quantitative polymerase chain reaction. For this, stably expressed reference genes are crucial. However, no studies on the stability of reference genes in THP-1 cells stimulated with LPS and INF- γ have been made. Therefore, this paper describes the selection of the most used reference genes (ribosomal protein L37a, glyceraldehyde-3-phosphate dehydrogenase, ubiquitin C, β 2-microbulin, β -actin and cyclophilin A), the in silico primer design, the analysis, and the validation of these in accordance with the MIQE guidelines and more recent recommendations for validation of the stability of reference genes. Using the RefFinder platform, including the four most popular algorithms for reference gene validation, the Delta CT, BestKeeper, NormFinder and geNorm, we find the reference genes GAPDH and UBC to be the most stable. Furthermore, we demonstrate that the normalization of gene expression data using the least stable reference genes, ACTB and B2M, dramatically affects the interpretation of experimental data. Taken together, it is vital to validate the stability of reference genes under the specific experimental conditions used when utilizing the THP-1 monocyte model system.

Method:

Phorbol 12-myristate 13-acetate (PMA) was applied to stimulate the differentiation of THP-1 monocytes into macrophage-like cells. Furthermore, lipopolysaccharide (LPS) and/or interferon- γ (IFN- γ) were employed to stimulate the THP1 macrophage-like cells towards a macrophage M1 phenotype. RT-qPCR was used to assess the differentiation process by the quantity of gene expression levels. As normalizing the stably expressed reference genes is critical to giving a robust and

sensitive detection degree of gene expression levels; hence we selected and validated six of the most used reference genes: ribosomal protein L37 (*RPL37A*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ubiquitin C (*UBC*), β 2-microbulin (*B2M*), β -actin (*ACTB*) and peptidylprolyl isomerase A (*PPIA*).

We employed the RefFinder, which allows for the four different algorithms, namely the Delta CT, NormFinder, BestKeeper, and geNorm, to rank the stability of the reference genes across all experimental conditions. Furthermore, we validated the most stable reference gene and least stable reference gene for each experimental condition by normalizing the expression of the two GOIs, Tumor necrosis factor-alpha (*TNFA*) and C-C Motif Chemokine Ligand 2 (*CCL2*).

Results:

- 1) When using the RefFinder algorithm on data from all experimental conditions, we observed that the most stably expressed genes in descending order were *GAPDH*, *UBC*, *RPL37A*, *PPIA*, *B2M* and *ACTB* (n=3 represents biological replicates, see appendices manuscript 3, Fig 3A).
- 2) We demonstrated that only when normalizing the expression of *TNFA* and *CCL2*, to the most stable reference genes, *GAPDH* and *UBC*, did we get a robust and statistically significant increase in cells stimulated with LPS + IFN- γ compared to LPS alone. (n=3 represents biological replicates, see appendices manuscript 3, Fig 4A and B).
- 3) We observed a decreased expression of *TNFA* and *CCL2* in the CM group when assessing THP-1 cells stimulated with LPS + IFN- γ alone or combined with CM (n=3 represents biological replicates, see appendices manuscript 3, Fig 4C and D). The decrease was most obvious when normalizing to the most stable reference genes, and for *TNFA*, the reduction was not statistical significance when normalizing to the least stable reference genes.

3.4 STUDY 3

► Cells. 2022 Apr 6;11(7):1236. doi: 10.3390/cells11071236.

Distinct Dominant Lineage from In Vitro Expanded Adipose-Derived Stem Cells (ASCs) Exhibits Enhanced Wound Healing Properties

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Affiliations + expand

PMID: 35406800 PMID: PMC8998068 DOI: 10.3390/cells11071236

Free PMC article

Abstract

It has been suggested that immunophenotypically defined lineages within the in vitro expanded adipose-derived stem cell (ASC) may play a beneficial role from the perspective of a personalized intervention. Therefore, to better understand the implications of different surface marker profiles for the functionality, we set out to examine the evolution of ASC-variants based on the co-expression of five bright or eight dim epitopes. At passages P1, P4, and P8, the co-localization of five bright markers (CD73, CD90, CD105, CD166, and CD201), or eight dim markers (CD34, CD36, CD200, CD248, CD271, CD274, CD146, and the Stro-1), was investigated by flow cytometry. Selected subpopulations were isolated using the fluorescence-activated cells sorting from the cryopreserved P4 and analyzed in terms of proliferative and clonogenic properties, trilineage differentiation, and wound healing potential. Only two of the dim epitopes were found in representative subpopulations (SP), and from the P4 onwards, two major combinations featuring the CD274⁺ (SP1) or the CD274⁺ CD146⁺ (SP2) emerged. Upon sorting and growth, both subpopulations assumed new but highly similar clonal profiles, consisting of the CD274⁺ CD146⁺ and the CD274⁺ CD146⁺ CD248⁺ phenotypes. The functional analysis revealed that the SP2 surpassed SP1 and the unfractionated cells regarding the growth rate, clonogenic activity, and the wound closure and endothelial tube formation potential. The surface epitopes may be considered a tool to enrich specific functionality and thus improve therapeutic outcomes in dedicated circumstances.

Method:

We comprehensively selected and sorted two representative ASC- subpopulations, CD274⁺ (SP1) and CD274⁺ CD146⁺ (SP2). We analyzed their characteristics and function variability based on a panel of quantitative characteristics and wound healing parameters, including the trilineage differentiation, proliferation, colony-forming unit capacity, and effect on fibroblast migration and endothelial tube formation capacity.

Results:

We revealed for the first time that the CD274+CD146+ subpopulation of ASCs had increased wound closure capacity and endothelial tube formation potential. Our study facilitates the broader use of purified immunophenotypical ASC subsets by exhibiting their improved ability.

3.5 STUDY 4

Evaluation of pentaisomaltose (PIM) as a cryoprotectant for adipose-derived stem cells

Qiuyue Peng, **Guoqiang Ren**, Vladimir Zachar, Simone R. Porsborg, Trine Fink. (Co-first author)

In preparation.

Background:

Adipose-derived stem cells (ASCs) have been widely explored to treat various diseases in many early clinical trials, where their safety and efficacy have been tested and shown great promise. A prerequisite for ASC-based therapies is the ability to cryopreserve the cells safely and efficiently during production or storage before treatment. Dimethyl sulfoxide (DMSO) has been commonly used as the golden standard cryoprotectant agent (CPA). However, DMSO has exerted toxic side effects on cells, primarily dependent on the DMSO concentration and cell exposure time throughout the pre-freezing and post-thawing phases.

Method:

ASCs were isolated, resuspended in CPA, and immediately frozen in liquid nitrogen (0h delayed group) or exposed for two hours at room temperature (2h delayed group). CPAs investigated were: HPL with either 5%, 2.5%, 1%, or 0% DMSO, or combined with 20% PIM (named PIM3, PIM2, PIM1 and PIM0, respectively). The effects of CPA on ASCs were analyzed through their trilineage differentiation capacity, surface marker phenotype, cell proliferation, cell viability, and proportion of CFUs.

Results:

- 1) The trilineage differentiation assessment demonstrated that all cultures have the ability to differentiate into adipocytes, osteocytes, and chondrocytes (see appendices manuscript 5, Fig 2).

- 2) The flow cytometric analysis of surface markers showed no significant differences between the various cryopreservation media groups (see appendices manuscript 5, Table 1).
 - 3) When evaluating proliferation, the results showed that freezing media PIM2 was superior to DMSO 2.5 % alone in both 0h and 2h delayed groups (n=3 represents biological replicates, see appendices manuscript 5, Fig 1 A1 and A2).
 - 4) Our results indicated that freezing media PIM2 was comparable to commercial media with 5% DMSO in both 0h and 2h delayed groups when assessing viability (n=3 represents biological replicates, see appendices manuscript 5, Fig 1B).
 - 5) The colony-forming potential was higher in all 2h delayed groups except for DMSO 1% when compared to 0h delayed groups (n=3 represents biological replicates, see appendices manuscript 5, Fig C).
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CHAPTER 4. DISCUSSION

Adipose-derived stem cells are a promising candidate for the treatment of chronic wounds. However, several challenges must be solved before they can be widely used for clinical practice.

In light of this, we proposed two hypotheses in this dissertation:

1. A panel of potential potency assays and markers relevant to wound healing are essential for selecting the best donors and purified ASC subsets for future manufacturing of ASC-based medicinal products.
2. The in vitro procedures of expansion and storage are essential for developing clinically relevant ASC-based medical products.

The potential potency assays and markers for selecting the best donors for treating wound healing

The first issue is the choice of fatty tissue as the source for ASCs, as it has been shown that ASCs from different donors vary significantly in characteristics and potencies. Thus, it is imperative to identify donors from which ASCs are most suitable for wound healing therapies by screening the ASCs in potency assays relevant to wound healing [132].

A sequence of preclinical studies has revealed the inconsistency between ASCs obtained from different donors in connection with proliferation and differentiation capacity [82–84,133,134]. Therefore, to bolster the success rate of implantation and therapy for future clinical use, the medicinal capacity of ASCs concerning wound healing and their inter-donor variability should be investigated more thoroughly.

In the Review manuscript, we proposed a panel of potential potency assays relevant to wound healing to incorporate in the future production of ASC-based medical products [121]. Due to the donor-dependent variability of ASC-based products, their quality should be verified utilizing a matrix of several of the most related potency assays. However, it was noted that the role of the single surrogate factors and their relationship to the overall clinical treatment effect urgently needed to be confirmed

[121]. Besides, attention should be paid when selecting the cell type for the functional assays. The primary cells are the preferred choice since they provide more relevant results and mimic the actual processes better than cell lines [135].

Based on this review, we investigated in Study 1 the discrepancy of ASC from different donors in terms of a set of characteristic and functional assays associated with stem cell characteristics and their MoA within wound healing [136]. The ASCs from all donors could undertake tri-lineage differentiation, possess a high proportion of CFUs and were able to proliferate. Furthermore, all three cultures were positive for the characteristic markers CD105, CD73, CD90, and our selected ASC markers CD146 and CD34. Moreover, a deficient expression of CD31 was noticed. It is worth noting that we present our marker's expression using the proportion instead of the mean fluorescent intensity (MFI), as the MFI only holds for normal distributions [137], but our data, such as CD271, CD146 and CD36, belong to bi-modal populations.

Additionally, we found substantial discrepancies for several markers compared to published research. For example, while the CD34 was initially not identified on ASCs [138], and some authors later only detected it in the early stages after primary isolation [139–142], our experiments revealed a robust expression of around 10% for all three donors (Supplemental Fig.1, The top 2.5 percentile of the FMO control was established for the positive population). Therefore, the expression of CD34 on ASCs may count on the cell passage number and the culture conditions utilized.

Based on the functionality of the ASCs obtained from individual donors, ASC-105 and -106 exhibited satisfactory attributes compared to ASC-101, which could be clarified by their proliferation capability or other functional properties. When investigating the effect of ASCs on target cells, different methods can be used as co-culture, transwell or conditioned media [143]. To simplify the assay for future routine use, we chose to use CM-based assays. To ensure a comparable number of ASCs between CM-productions, we seeded the cells at the same density for each group, ensured complete cell attachment after 24 hours, and then collected the conditioned medium (CM) for the functional assays. Other ways to ensure comparability between CM productions are to

quantify the amount of DNA, count the cells in the culture after CM harvest, or quantify the total protein content in the CM [144]. No matter which method is used, it should be easily integrable into routine testing.

To get closer to the MeA of ASCs in wound healing, the regulation of genes involved in processes related hereto was examined by RNA sequencing (RNA-seq) and gene ontology analysis. Our analysis revealed that stem cell- and wound-healing-related genes primarily related to ECM and angiogenesis are involved. Based on their biological significance and the level of available evidence by a thorough literature review, we propose a panel of stem cell- and wound-healing-related genes, especially those connected to ECM and angiogenesis, which could be employed as markers of a superior ASC donor to hire ASCs for treating chronic wounds [134]. The suggested panel comprises 10 genes of particular interest coding for transcription factors (EPAS1, TBX1), membrane proteins (ITGB8, FGFR2, GREM1), and secreted proteins (MMP1, MMP9, COL4A4, CCL11 and FGF9). However, as mentioned, ASCs play a positive role in the wound healing process mainly through their secretome [40,44,48–57,60–80]. Thus, the genes coding for secreted proteins might be of special interest when identifying ASC functional attributes associated with wound healing.

MMP1, MMP9, and COL4A4, have all been indicated to play a crucial role in angiogenesis. MMP1 has been observed to initiate vascular remodeling and angiogenesis by promoting endothelial cell proliferation and enhancing the vascular endothelial growth factor receptor 2 expressions in the wound healing process [145]. MMP9 has been proven to accelerate VEGF secretion from endothelial cells and motivate vessel growth by enhancing endothelial cell migration [146]. COL4A4 encodes the alpha4 (IV) chain of type IV collagen. Type IV collagen is a critical element of the endothelial basement membrane to maintain the formation of blood vessels and facilitate endothelial cell migration [147,148]. In addition, it has been demonstrated that MMP1 and MMP9 play a role in wound healing by contributing to fibroblast proliferation and migration [149–152]. FGF9 is essential in wound healing, as evidenced by increasing migratory fibroblast capacities and stimulating angiogenesis [153–159]. CCL11, also known as eotaxin-1, as evidenced by its ability to accelerate the migration and proliferation of fibroblasts and endothelial and angiogenesis [160–162]. These upregulated genes

could be indicated as potential screening markers for ASCs with enhanced property concerning wound healing.

In our study, discrepancies in the expression of *PECAMI*/CD31 were found between mRNA and surface marker levels. *PECAMI*, also known as CD31, is essential in endothelial biology and angiogenesis [163]. We observed that mRNA expression of *PECAMI* was upregulated in ASC-101 compared to ASC-105 and -106, but that the percentage of CD31+ ASCs was less when measured by flow cytometry. Firstly, this inconsistency could be caused by the two different technologies' detection methods, thus making the mRNA expression inconsistent with the surface marker levels. Moreover, the mRNA expression of *PECAMI* between ASC-101, -105, and -106 is relatively low (FPKM value: 0.92, 0.3, 0.26, respectively), which could mask the actual biological difference between donors.

The potential reference genes for qPCR of differentiation and maturation of THP-1 cells to M1 macrophage-like cells

Macrophages play a vital role in the inflammation process, and two subtypes of macrophage have been found, thereinto, M1 macrophages are pro-inflammatory phenotype and responsible for the initial inflammatory stage, while M2 is the anti-inflammatory subset that contributes to the resolution of the inflammatory process [164]. These two phenotypes are distinguished based on their phenotype and distinct gene expression pattern [164,165]. To investigate the effect of ASCs and the difference between donors on the inflammation phase and these macrophage phenotypes, qPCR is a widely used method for quantification. However, no consensus exists on using specific reference genes in this area. Therefore, we showed in Study 2 the significance of assessing potential reference genes to interpret biological reactions based on the experimental parameters selected [166].

When exploring factors that might impact monocyte and macrophage function and biology, THP-1, an immortalized monocyte cell line, has been acknowledged as a reasonable alternative to primary monocytes and macrophages to avoid interdonor variation [167,168]. Phorbol 12-myristate 13-acetate (PMA) is generally applied to stimulate the differentiation of THP-1 monocytes into macrophage-like cells.

Furthermore, lipopolysaccharide (LPS) and/or interferon- γ (IFN- γ) are commonly employed to stimulate the THP1 macrophage-like cells towards a macrophage M1 phenotype [169,170]. To assess the differentiation of THP-1 monocytes into macrophage-like cells and M1 phenotype is broadly achieved by the quantity of gene expression levels by RT-qPCR. To give a robust and sensitive detection degree of gene expression levels, RT-qPCR depends on normalizing the gene expression data to one or more stably expressed reference genes [171]. Maeß, M.B. et al. have reported that the *ACTB* and *RPL37A* were the most stable reference genes when promoting THP-1 monocytes differentiation into macrophages induced by the PMA [172]. Cao X et al. indicated that *PPIB* and *PGK1* were the most stable reference genes in a study of the LPS-stimulated THP-1 cell model [173], and Kalagara R et al. reported that *UBC* and *B2M* were the most stable genes to normalize RT-qPCR data from LPS-stimulated macrophages [174]. However, no studies have focused on selecting reference genes for the polarization of THP-1-derived macrophages to M1 macrophages.

As a result, we selected six widely used reference genes from the literature, to which we designed primers and ranked in the sequence of stability across a variety of THP-1 differentiation and polarization protocols using five various algorithms, the RefFinder, Delta CT, Best-Keeper, NormFinder, and geNorm [124,125,175–179]. We observed the degressive sequence of stability of the genes to be *GAPDH*, *UBC*, *RPL37A*, *PPIA*, *B2M*, and *ACTB* [172,180–183]. To understand the maturation and polarization of THP-1 cells, we preferred two M1 macrophage polarization markers, *TNFA* and *CCL2* [184–186]. We demonstrated that only when normalizing the expression of *TNFA* and *CCL2*, to the most stable reference genes, *GAPDH* and *UBC*, did we get a robust and statistically significant upregulation in cells cultured with LPS + IFN- γ compared to LPS alone. Besides, we evaluated the effect of conditioned medium from ASCs on the differentiation and maturation of THP-1 cells to M1 macrophage-like cells to find that ASC's anti-inflammatory properties could be masked when normalization of gene expression data using the least stable reference gene is performed. Thus, when normalizing gene expression data for the gene of interest, careful evaluation of reference gene stability must be exercised, especially when studying the responses of cell types, for instance macrophages,

which are highly reactive to even delicate changes in the inflammatory setting of their milieu [166,171].

Therefore, the stability of reference genes should always be validated for studying the responses of particular cell types under specific experimental conditions. Firstly, a literature review should identify several potential reference gene candidates. Following, RT-qPCR should be employed to test the stability of several candidate reference genes under individual experimental conditions. Ultimately, two or more algorithms ought to be applied to distinguish the most stable reference gene.

The potential of purified ASC subsets for wound healing therapies

The regularly used isolation approach for ASCs employs the enzyme digestion of adipose tissue, after which ASCs are purified by their plastic adherence capability. However, more and more focus is on selecting and isolating specific subpopulations with higher potency than crude ASCs. Despite inherently maintaining a typical phenotype distribution, the discrete ASC subpopulations have shown differential functional properties after in vitro expansion [187]. It has been proposed that immunophenotypically defined lineages within in vitro expanded ASC may play a positive role from the view of dedicated circumstances [187,188]. Nielsen, F.M. et al. reported that the ASC subpopulation (CD73+90+105+34-146+271-) could provide a superior wound healing potential for relevant cell-based medical products [187]. Traktuev, D O et al. demonstrated that the CD34+CD146+ phenotype of ASC could have a highly pro-angiogenic property [142]. However, more studies are required to shed more light on the biological significance of ASC subsets.

To better understand the connotations of different surface marker profiles in the wound healing perspective, we examined in Study 3 the evolution of ASC-variants based on the co-expression of CD markers [189]. The two most archetypal lineages, CD274+ (SP1) and CD274+CD146+ (SP2) were purified and analyzed for their characteristics and function in wound healing. Our study revealed that ASC from the purified subpopulations (SP1 and SP2) was superior in proliferation, clonogenic potentials, adipose/osteogenesis, and wound healing capacity compared to the unsorted ASC cultures. It could be presumed that stem

potentials of heterogeneous cells were inhibited after contamination with other populations of progenitors or precursors [190,191]. Our study facilitates the broader use of purified immunophenotypical ASC subsets by exhibiting their improved ability.

Moreover, we found that the SP2 (274⁺146⁺ sorted subpopulation) demonstrated superior wound healing properties in terms of fibroblast proliferation and tube formation of endothelial cells compared to SP1(274⁺ sorted). Supporting CD146 as being broadly identified as linked to pro-angiogenic effects in MSCs [192–195]. Likewise, the 146⁺ sorted ASCs have indicated favorable angiogenic properties by secreting VEGF, FGF, and angiopoietin-1 [196,197]. This indicates that more work should be poured into CD146 to shed more light on the implication of distinct immunophenotypes for evaluating ASC biotic potential.

The potential new cryoprotectant for ASCs cryopreservation

Effective methods for cryopreservation of ASCs are critical to retaining the maximum viability and function of the ASCs before clinical use. Among various CPAs, 10% DMSO and FBS are the most widely used intracellular cryoprotective agents for the cryopreservation of cells and tissues, including ASCs [198–200], preventing the formation of intracellular and extracellular crystals. However, DMSO is recognized to exert toxic side effects on cells, so a series of new DMSO-free CPAs have been developed. To replace DMSO, polyvinylpyrrolidone (PVP), trehalose, and methylcellulose have been used as new CPAs [129,201,202], however, they are less effective than DMSO in terms of sustaining ASC viability, recovery, and potency. PIM, a potential new CPA, has been approved for clinical use, but the cryoprotective effect of PIM on ASCs remains poorly examined. In Study 4, we investigated the cryoprotective effect of PIM as an alternative to DMSO for ASCs cryopreservation.

As described in the result section, ASCs could be effectively cryopreserved in a freezing medium combining PIM with a low DMSO concentration, reducing the use of DMSO to only 2.5%, which could be an efficient and less toxic CPA combination. As DMSO has concentration-

dependent cell toxicity, lowering the DMSO concentration may be an ideal option. The effectiveness and safety of PIM have been examined in the cryopreservation of T cells and peripheral blood / hematopoietic stem cells [127,203,204]. A recent study established that ASCs could be effectively cryopreserved in the freezing media containing PIM and DMSO, and the addition of PIM allows for reducing the DMSO concentration to 2% when only evaluating the post-thaw viability, which was consistent with our results [131]. However, we indicated that ASCs cryopreserved with 20% PIM alone showed a reduced colony-forming potential compared to 2.5% or 5% DMSO or PIM combined with either 2.5% or 5% DMSO. It is presumed that the additive effect exists when a permeating CPA (DMSO) is combined with a nonpermeating CPA (PIM). Additionally, the concentration of PIM used as CPA may depend on the cell type, with the concentrations ranging between 10% to 30% [127,131,203,204]. Factors such as cell surface area to volume ratio and the penetrability of cytomembrane to water may cause variations [131,205].

3.3 LIMITATION

For Study 1, it should be mentioned that this study explored the ASC-donor variability employing the RNA-Seq approach only to emphasize the distinctions in the gene regulation in ASCs between different donors, which could be applied to clarify the observed difference in effect [136]. Additional research based on multiple validations is expected to shed more light on the biological distinction among donors in wound healing and entirely confirm these markers as the MeA of ASCs in wound healing [136]. In addition, we propose a panel of the molecular targets identified from the RNA-seq that were thoroughly reviewed by a literature search to uncover available evidence of their MeA within stem cell function and wound healing, and, based on this, they should be narrowed down to a suggestive panel for further analysis.

Another concern is the choice of the wound healing model used in Studies 1 and 3. We used simplified 2D in vitro wound healing models to assess the ability of ASCs in this regard. It should be pointed out that the cells in vitro models are harvested from their ecological niche and exposed to conditions extremely distant from the biological ones. Thus, the in vitro difference in the cell's ecological environment may lead to

abnormal cell behavior compared to those normally residing in situ, which will mislead the actual ability of ASCs. Therefore, in vivo models might depict the intervention's process and effect more realistically.

For study 2, a plethora of M1 markers have been suggested in the literature. Some have been used in the past, some very sparsely, and others more broadly. To increase the impact of this paper, we analyzed the use of M1 markers and concluded that *TNFA* and *CCL2* were the most representative M1-related markers. We believe, for the message of this paper, that these two representative markers can explain the importance of reference gene selection for the researcher under the specific experimental conditions used when employing the THP-1 monocyte model system, but that including more markers could strengthen this message.

The last consideration is the non-negligible effect of donor interpersonal differences. Study 3 indicates that selected clones with enhanced wound-healing potential may exist within the expanded ASC subsets. However, it is still unknown how universally this phenomenon applies to the donor population; therefore, continuation studies are required in this area to promote more efficient and personalized ASC-based therapies [189].

GUOQIANG REN

TRANSLATION OF ADIPOSE-DERIVED STEM CELLS INTO CLINICAL USE TO TREAT CHRONIC WOUNDS

CHAPTER 5. CONCLUSION

In conclusion, this dissertation supports a better understanding of the heterogeneity between ASC from different donors, which would boost the translation of ASCs into clinical applications.

The results presented in Studies 1 and 3 suggested that a panel of ASC markers relevant to wound healing may serve as guidelines for selecting the best donors or purified ASC subsets for future manufacturing of ASC-based medical products.

In study 2, we showed the significance of assessing potential reference genes to explain biological reactions when differentiating the THP-1 monocytes into the M1 phenotype, and ASC's anti-inflammatory properties could be masked when normalization of gene expression data using the unstable reference gene is performed.

In study 4, we demonstrated that ASCs could be effectively cryopreserved in a freezing medium combining PIM with a low DMSO concentration, reducing the use of DMSO to only 2.5%, thus, suggesting a better choice of CPA for the benefit of ASCs as an off-the-shelf product for future use.

Together, our findings ensure a better understanding of ASCs and the practical handling of these, inspiring the approaching translation of ASCs into clinical use for treating chronic wounds.

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TRANSLATION OF ADIPOSE-DERIVED STEM CELLS INTO CLINICAL USE TO TREAT CHRONIC WOUNDS

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APPENDICES

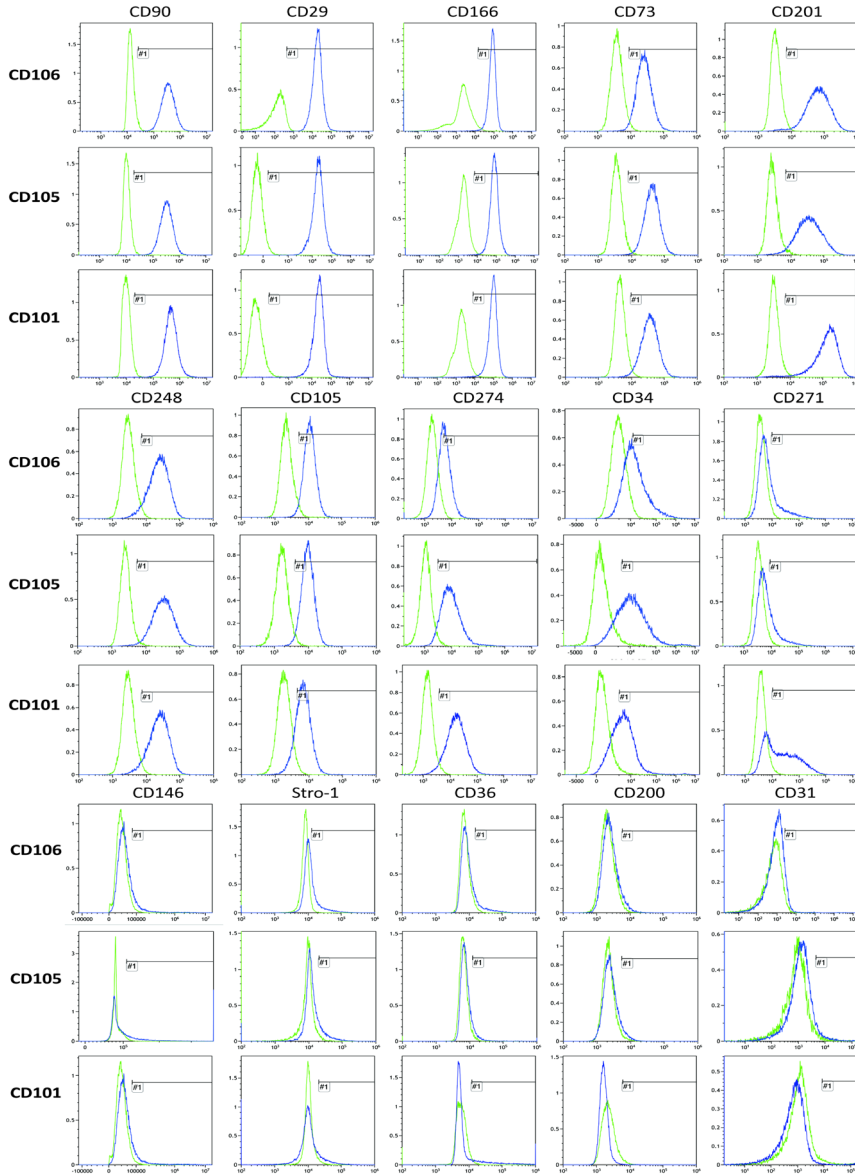


Figure S1: Histogram of individual surface markers across donors. Cells from 3 donors were harvested and proceeded to conduct flow cytometry independently. The top 2.5 percentile of the FMO control was established for positivity (bar gate). The green histogram represents FMO control, and the blue represents the stained sample. Abbreviation: D, donor; FMO, fluorescence minus one.

GUOQIANG REN

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