



Review

Advances in regenerative therapy: A review of the literature and future directions

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ABSTRACT

There is enormous global anticipation for stem cell-based therapies that are safe and effective. Numerous pre-clinical studies present encouraging results on the therapeutic potential of different cell types including tissue derived stem cells. Emerging evidences in different fields of research suggest several cell types are safe, whereas their therapeutic application and effectiveness remain challenged. Multiple factors that influence treatment outcomes are proposed including immunocompatibility and potency, owing to variations in tissue origin, ex-vivo methodologies for preparation and handling of the cells. This communication gives an overview of literature data on the different types of cells that are potentially promising for regenerative therapy. As a case in point, the recent trends in research and development of the mesenchymal stem cells (MSCs) for cell therapy are considered in detail. MSCs can be isolated from a variety of tissues and organs in the human body including bone marrow, adipose, synovium, and perinatal tissues. However, MSC products from the different tissue sources exhibit unique or varied levels of regenerative abilities. The review finally focuses on adipose tissue-derived MSCs (ASCs), with the unique properties such as easier accessibility and abundance, excellent proliferation and differentiation capacities, low immunogenicity, immunomodulatory and many other trophic properties. The suitability and application of the ASCs, and strategies to improve the innate regenerative capacities of stem cells in general are highlighted among others.

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Abbreviations

ADSCs	Adipose tissue-derived stromal cells	ITS	Insulin-Transferrin-Selenium
ASCs	Adipose stem cells	iPS	Induced pluripotent stem cells
b-FGF	Beta-fibroblast growth factor	iTS	Induced Tissue-specific stem cells
BM-MSCs	Bone marrow-derived mesenchymal stem cells	MCP	Monocyte chemoattractant protein
CCL	Chemokine ligand	MIP	Macrophage Inflammatory protein
CFU-F	Colony forming unit fibroblast	MMP	matrix metalloproteinase
CS	Cell sheet	mRNA	Messenger Ribonucleic acid
DSCs	Placenta derived decidua stromal cells	MSCs	Mesenchymal stromal cells
ECM	Extra cellular matrix	PAI	Plasminogen activator inhibitor
ESCs	Embryonic stem cells	PDGF	Platelet-derived growth factors
HLA	Human leukocyte antigen	Pdx1	Pancreatic and duodenal homeobox 1
HNF4 α	Hepatocyte nuclear factor 4 alpha	SCs	Stem cells
HSCs	Hematopoietic stem cells	TF	Tissue factor
IFN γ	Interferon gamma	TGF- β	Transforming growth factor beta
IGF	Insulin growth factor	TNF- α	Tumor necrosis factor-alpha
IL	Interleukin	UCB-MSCs	Umbilical cord blood mesenchymal stem cells
		VEGF	Vascular endothelial growth factor

1. Introduction

Regenerative therapy is the therapeutic application of stem cells and/or progenitor cells based on their potential to stimulate repair mechanisms and restore function in damaged body tissues or organs [1–4]. Stem cells (SCs) are defined by their self-renewal and differentiation capacity into one or multiple specialized cell types [5,6], as well as their unlimited regenerative potential through various trophic properties [7–9]. SCs are classified into two broad categories according to their differentiation capacity and tissue of origin (Table 1). SCs are referred to as totipotent, pluripotent, multipotent or unipotent cells depending on their differentiation ability [10,11]. Totipotent cells are those capable of giving rise to a new organism as they can differentiate into all tissues including germline and extra-embryonic tissues. Pluripotent cells are able to generate all body cells including germ cells. Multipotent cells are capable of generating all or at least some tissue cell types. Unipotent cells are able to generate a single cell type. The classification according to origin is essentially divided into two main cell types: embryonic stem cells (ESCs) [12] and tissue derived (somatic) stem cells [13,14]. The tissue derived stem cells are further divided into: fetal stem cells isolated from fetal tissues [15] such as placenta [16], amniotic fluid [17], Wharton jelly [18], umbilical cord blood [19], adult stem cells isolated from various tissues including bone marrow (BM-MSCs) [20], adipose tissue (ASCs) [8], dental pulp [21], skeletal muscle [22], skin [23], peripheral blood [24], liver [25], neural tissue [26], heart [27], and intestine [28]. Recently, two more sub-divisions of tissue-derived stem cells have been reported: induced pluripotent stem (iPS) cells [29–32] and induced tissue-specific stem (iTTS) cells [14,33,34].

This review describes several important aspects of each SC category based on their origin, and offers greater emphasis on adult stem cells. The adult stem cells also known as multipotent mesenchymal stromal/stem cells (MSCs) have been extensively studied for over three decades for their therapeutic potential over a

wide range of diseases. A plethora of preclinical studies have demonstrated the consistent ability of MSCs to promote tissue healing, reduce excessive inflammation and improve outcomes in a wide range of animal disease models [35]. However, human clinical translation in advanced phases present variable and discordant outcomes. Therefore, deciphering the reasons of dissonance is indeed paramount. The currently proposed factors contributing to the differences between animal model findings and clinical outcomes include inter alia differences in the preparation, potency, and functionality of MSCs in terms of tissue source, culture, and expansion [35]. ASCs are particularly promising candidates for diverse clinical applications, owing to their excellent proliferation and differentiation capacity [8,36], low immunogenicity [37,38], and ability for immunomodulation [37,39–43]. Here, the clinical suitability of MSCs is highlighted in detail while focusing more on current applications, benefits, challenges, and strategies to improve the therapeutic efficacy of stem cells.

1.1. Embryonic stem cells

Embryonic stem cells (ESCs) are pluripotent cells with the ability to differentiate into any mature cell types of the trilaminar germ lines. ESCs are obtained from the inner cell mass of the early (5–7 days post-fertilization) pre-implantation blastocyst. They were initially derived from mouse embryos in the early 1980s, and later from a number of different species including rat, rabbit, sheep, pig, horse and human [12]. Human ESCs are promising candidates for cell-based therapy given their distinctive properties such as; self-renewal, pluripotency and genomic stability [44]. At the beginning of the 21st century, ESCs generated great interest in different fields namely regenerative medicine, immunotherapy, and drug discovery. However, application of these cells is challenged by the limited access to the tissues of origin. Moreover, they are currently considered high risk because of their potential to form teratomas,

Table 1
Classification of stem cells according to differentiation capacity and origin.

Differentiation capacity	Properties	Notes
Totipotent	Differentiation into embryonic and extraembryonic cell types, Capacity to form complete, viable organism	Result from fusion of egg and sperm and initial fertilization of egg
Pluripotent	Differentiation into nearly all cells of trilaminar germ layers: ectoderm, endoderm and mesoderm	Descendants of totipotent cells Examples include Embryonic stem cells and Induced tissue derived stem cells
Multipotent	Differentiation into a number of particularly closely related cell types.	Examples include Hematopoietic stem cells and Mesenchymal stem cells
Oligopotent	Differentiation into only a few cell types	
Unipotent	Self renewal and proliferation, but produce own cell type only	Example: Spermatogonial stem cells
According to origin	Sub-types	Notes
Embryonic stem cells		<ul style="list-style-type: none"> - Derived from inner cell mass of a blastocyst, - Possess distinctive self-renewal, pluripotency and genomic stability properties - Are potentially teratogenic - GMP production generally challenged - Have ethical restrictions [12,15,16,47–50]
Tissue derived stem cells	Fetal stem cells	Fetal: Fetal blood, Bone marrow, etc. Extra fetal: Placenta, Amniotic fluid, Wharton's Jelly, Umbilical cord (UMSCs) Decidua basalis, Decidua parietalis
	Adult stem cells	Induced pluripotent stem cells (iPS) can be derived from all tissues Induced tissue-specific stem cells (iTS) Mesenchymal stem cells (MSCs)
		iPS: <ul style="list-style-type: none"> - Generated by overexpressing embryonic genes; Oct4/3, Sox2, Klf4, and c-Myc - Have similar properties of ESCs at cellular level - Envisaged increase in usage in the elucidation of stem cell and disease pathways as well as personalized drug discovery - Clinic use remains challenged with cost and standard of production, and questionable safety due to potentials of teratoma formation [32–35,51–55,73] iTS: <ul style="list-style-type: none"> - Generated by transient overexpression of the reprogramming factors - Thought to be superior in differentiation and lack concerns of teratoma formation [14,33,34] - derived from vascularized tissues such as: Bone Marrow, Adipose tissue, Skin, Periodontal tissue - Sources like adipose are readily available and easier to access with minimal restrictions - Multi-potent, self-renewing cells - can be induced both invitro and <i>in vivo</i> into multilineage differentiation - Have angiogenic, immunomodulatory, inflammatory and apoptotic properties [11,22–26,38,67,85–88,94,95]

the difficulty in obtaining clinical grade quality cells and the restrictive ethical concerns [9,13,45–47].

1.2. Tissue derived stem cells

1.2.1. Induced pluripotent stem cells

During the period of 2006–2009, three independent research groups namely, Shinya Yamanaka [29], James Thomson [48], and George Q. Daley [49] have reported successful genetic reprogramming of somatic cells to stem-like cells and coined the term induced pluripotent stem cells (iPS). The Nobel laureate Yamanaka and his group were the first to successfully reprogram mouse embryonic fibroblast cells in 2006 [29], a year later human skin fibroblast derived iPS cells were reported [31,48,50], prior to the use of peripheral blood mononuclear cells as a tissue source [49].

iPS cells are generated from adult cells by overexpression of embryonic genes or transcription factors named “Yamanaka factors” including Oct4/3 (octamer-binding transcription factor 4/3), Sox2 (sex determining region Y)-box 2 (sex determining region Y), Klf4 (Kruppel-like factor 4) and c-Myc (Avian Myelocytomatosis virus oncogene cellular homolog) [29–32]. At the cellular level iPS cells are almost identical to ESCs because of their inherent abilities

to self-renew, proliferate and produce germ line competent-chimeras. iPS cells have the additional advantages of easy accessibility and expandability, and that they can be induced to differentiate into hundreds of cell types [51,52]. Moreover, iPS cells are derived from adult cells, not embryos, overcoming major ethical restrictions to use. Armed with such properties, iPS cells have in the recent past notably contributed to advances in stem cell biology and regenerative medicine, especially in the direction of personalized medicine. Also, the iPS cell technology has incorporated innovative technologies such as gene editing and three-dimensional organoids, which have greatly boosted efforts in disease modeling, drug discovery, and cell therapy [53,54]. Notwithstanding, even with the integration of such methodologies and technologies, differentiation to target cells remains a challenge [45,55–57]. With existing reports of stem cell lines acquiring certain genetic alterations that are associated with human cancer [58], both genomic stability and human clinical translation of iPS cells remain areas of contention. Some scientists urge caution with the use of iPS-based cell therapies in human trials, and challenge the need for genetically manipulated cells for the practice of medicine [59]. Indeed, some evidence promote a debate on concerns that iPS cells may exhibit tumorigenic features [31,48,60].

Moreover, the comparison of the transcriptomes of iPS cells with the transcriptomes of cancer cells has demonstrated that induced pluripotency and oncogenic transformation are related processes [61–63]. There is also the argument that naturally pluripotent stem cells exist and can be isolated without first being genetically modified [64–67]. The essence of these limitations lies in the fact that it takes a long time to achieve stable iPS cell lines due to their unlimited proliferation and inherent ability to form tumors. Relentless efforts are, however, intended to eliminate malignant transformation risks through the use of differentiated iPS cells. It is critical though that the populations of these differentiated cells are well defined and not contaminated [68]. Therefore, deciphering strategies to promote standards and guidelines to obtain large, well-defined and well-characterized sets of cells is of paramount importance [69]. However, such efforts are still in a developing stage; yet universally acceptable standard functional assays for selection of differentiation biomarkers has not been achieved.

Furthermore, because iPS-based cell therapies are geared for global application, allogeneic supplies are preferred amidst concerns inter alia immune reactions and infections. This is because the allogeneic approach can reduce both cost and time of production as it is possible to perform prior comprehensive quality testing and generate a sufficient number of clinical grade iPS cells. Against this backdrop, to reduce the risk of immune reactions the University of Kyoto Center of iPS Cell Research and Application (CiRA) has recently developed an approach of generating safe iPS stocks from healthy HLA homozygous donors, that maximizes matching of local consumer population at a major HLA locus [70]. However, solutions to other challenges including the development of animal disease models to appropriately recapitulate human disease for pre-clinical studies, lack of in-depth understanding of therapeutic mechanism of action, and the costs of clinical-grade iPS cell production and quality control that are significantly high are still highly sought after [56].

Therefore, owing to the existence of several unresolved issues related to the use of iPS cells for clinical therapies, particularly their potential for tumor formation and limited ability to generate pure populations of differentiated cell types *in vitro*, Noguchi Hirofumi of the Department of Regenerative Medicine, Graduate School of Medicine, University of the Ryukyus, and colleagues [14,33,34] have recently successfully generated tissue-specific stem cells and coined the term induced tissue-specific stem cells (iTSC). The iTSC cells were generated from mouse pancreas and liver by transient overexpression of the reprogramming factors using the transcription factors: pancreatic and duodenal homeobox factor-1 (Pdx1) and hepatocyte nuclear factor 4 alpha (HNF4 α). Their recent review describing the epigenetic memory phenomenon in iPS and iTSC cells, emphasizes the relative importance of iTSC to clinical application of stem cells, the superior differentiation capacity and the lack of teratoma formation [14].

1.2.2. Fetal stem cells

Fetal Stem Cells are multipotent and with minimal ethical restrictions [71]. The most intensively studied are hematopoietic (HSCs) and MSCs. Fetal MSCs can be harvested from fetal tissues such as blood and bone marrow or extra fetal (perinatal) tissues like placenta, amniotic fluid, Wharton jelly, umbilical cord blood, decidua basalis, and decidua parietalis [15–19]. Fetal MSCs have reportedly better intrinsic homing and engraftment ability, greater multipotency and lower immunogenicity compared to adult stem cells [46]. Notwithstanding, the harvesting, potential and clinical application of fetal MSCs remains controversial. Some studies consider the harvesting process relatively harmless, and do not clearly distinguish fetal and adult sources given that sometimes the origin can be both maternal and fetal [16,18,72,73]. MSCs from

perinatal tissues are morphologically and immunophenotypically similar to those from adult sources and some such as umbilical cord blood derived MSCs (UCB-MSCs) possess unique properties. For instance, Kern Susanne et al. [74], in their comparative analysis, reported that UCB-MSCs were superior to BM-MSCs and ASCs regarding culture length and proliferation capacity. UCB-MSCs can be expanded in higher numbers regardless of Isolation success rates, and can be stored for longer periods for both allogeneic and autologous use [75]. However, *in vitro* studies have reported contradictory results on the procoagulant effects of MSCs upon contact with blood both in animals and humans [76]. Some of the initial reports on the use of human placenta-derived MSC-like cells in a Phase 1b/2a study in Crohn's disease highlighted the occurrence of venous thrombotic events in two patients [77], and posited tissue factor (TF) expression by the clinical-grade therapeutic MSCs as a possible cause [78,79]. Furthermore, subsequent *in vitro* studies comparing placenta-derived decidua stromal cells (DSCs) to BM-MSCs found a 15-fold higher expression of TF in the DSCs [80]. Furthermore, a recent study reported thromboembolism in two patients treated with UCB-MSC products [81].

1.2.3. Adult stem cells

The biological history of adult stem cells dates back to 1909 with the discovery of HSCs in bone marrow [82]. These cells were first identified as multipotent in the 1970s by Alexander Friedenstein and colleagues [83]. They referred to a unique rodent bone marrow cell population with characteristics of plastic adherence and multilineage differentiation capacity as colony forming unit fibroblasts (CFU-F) [84]. In the early 1990s, CFU-F generated global interest in science and clinical practice, and became popularly known as “Mesenchymal Stem Cells (MSCs)” because of their multilineage differentiation capacity, immunomodulation, and regenerative abilities. MSCs have since become the most clinically studied experimental cell therapy platform, albeit with a mixture of successes and challenges [35,85]. Extensive investigations have consistently demonstrated MSCs as heterogeneous, nonclonal mix of multipotent stem cells, committed progenitors, and differentiated cells [86]. Hence, the MSCs nomenclature has been often contested, and indeed over time the cells have been called different names including “Marrow Stromal Cells,” “Multipotent Stromal Cells,” “Mesodermal Stem Cells,” and “Mesenchymal Stromal Cells.” Latterly, persistent discussions on the accurate description of the origin, developmental potential, and biological functions of MSCs have proposed that “Tissue-Specific Progenitor Cells” or “Medicinal Signaling Cells” as more appropriate terms [87–91]. Conceivably, the definition entailing bone marrow origin, plastic-adherence, and the two true stem cell criteria of self-renewal and differentiation potential provide for *in vitro* manipulation, in bulk and at single-cell level, with no clear description of the characteristics displayed by unmanipulated MSCs *in vivo*. Moreover, it has now been demonstrated that MSCs arise from pericytes; in fact, they can be isolated from almost every tissue that is vascularized including bone marrow (BM) [20], adipose tissue [8], umbilical cord [19], dental pulp [21], and skin [23]. These cells additionally function as paracrine and secretory centers at injury sites in the body [64,91,92].

In 2006, the Mesenchymal and Tissue Stem Cell Committee of International Society for Cellular Therapy (ISCT) published minimum criteria for the identification of MSCs [93]. ISCT recommends that MSCs should be plastic adherent, with more than 95% of the cell population expressing flow-cytometrically determined CD73/5'-nucleotidase, CD90/Thy-1, and CD105/endoglin. In addition, less than 2% of the population should express CD34, CD45, CD11b/integrin alpha M or CD14, CD 79 alpha or CD 19, and HLA class IIa. Also, the cells must demonstrate tri-lineage differentiation

potential into osteoblasts, adipocytes, and chondroblasts under *in vitro* conditions. Additionally, surface markers CD13, CD29, and CD44 should be constitutively expressed by >80% of ASCs, while CD31, CD45, and CD235a, which are primarily negative markers, should be expressed by less than 2% of cells [94]. Of note, however, currently no single surface marker is capable of identifying cells *in vitro* and *in vivo*, that satisfy the minimal criteria of MSCs from various tissue sources. A number of candidate markers possibly related to the stemness of MSCs *in vitro* including Stro-1, SSEA-4, CD271, and CD146 have been reported, but not yet conclusively established due to large differences in their expression in cells from various sources probably arising from the dynamic influence of various culturing factors [95–97].

Despite encouraging pre-clinical findings from a wide range of disease models, after more than three decades, the efficacy of MSCs in advanced clinical trials remains challenged. However, the quest for an efficacious MSCs population in the clinic has intensified. The promising evidence for MSCs therapeutic success is based on cells' relative ease of isolation and expansion in culture, multilineage differentiation capacity, immunomodulatory, anti-inflammatory, anti-microbial, and regenerative effects, homing and migratory capacity to injury sites, safety profile in allogeneic transplantation, and the few ethical restrictions [91,98–102]. However, deeper understanding of factors contributing to the biological and pharmacological disparities between pre-clinical research and human translational studies is needed. Suggested factors significantly contributing to the dissonance include differences in cell preparation, potency, and functionality among MSCs tissue sources, culture methods, and expansion levels, and variations in cell handling including thawing, route of delivery, and dosing [35]. Indeed different researchers have demonstrated that the yield, proliferation, differentiation, biological and therapeutic functions of MSCs are influenced by tissue of origin, method and site of harvest, cell isolation and clinical application procedures [103–105]. Among the tissue sources, BM and adipose tissue are the most studied and prevalent in the clinical trials of MSCs [35,106]. As summarized in Table 2, BM-MSCs exhibit significant potential for promoting tissue regeneration, protecting ischemic tissues at risk, and modulating inflammation and autoimmunity [107]. Indeed BM-MSCs could be the most promising among MSCs cellular therapies, but clinical implementation remains challenging [35]. A small fraction of true pluripotent stem cells exists in BM compared to an overwhelming proportion of HSCs [108,109]. Therefore, utilizing BM-MSCs for therapeutic purposes typically requires large amounts of BM.

Moreover, isolation and expansion of BM-MSCs in culture require careful monitoring and minimal passaging to avoid cell senescence. It has been demonstrated that senescence potentially affects efficacy of the treatment of diseases, for example intervertebral disc degeneration [110]. In addition, although considered a safe procedure, the aspiration of BM commonly from the sternum and posterior iliac crest has documented fatal complications [111]. However, adipose tissue is abundant in most individuals, and a small amount (25–100 ml) can be harvested using a simple liposuction procedure with minor adverse effects and yield orders of magnitude more MSCs per unit volume [95,112–114]. ASCs can maintain chromosomal stability in extended passaging [115], and are capable of exhibiting tri-lineage differentiation potential with unique expression of certain cell adhesion molecules [116,117]. Numerous preclinical studies support the safety and efficacy of ASCs for regenerative medicine [118,119]. Therefore, this review preferentially provides a more detailed account of the unique properties, clinical application and proposed further research of ASCs in the subsequent sections.

1.2.3.1. Adipose-derived stem cells. The initial demonstrations of animal adipose tissue derived stromal vascular (progenitor) cells and their potential to proliferate were provided in 1964 and 1977 by Martin Rodbell and Van and Roncari, respectively [120,121]. Later in 2001, Zuk and colleagues successfully isolated and characterized a population of MSCs from human adipose tissue [8]. Adipose tissue is an abundant source of ASCs for immediate and/or long term utilization and, therefore, an important source for cell based therapy and tissue engineering [122–124]. Processed adipose tissue yields a mixture of stromal and vascular cells known as stromal vascular fraction (SVF), and ASCs derive from further processing of SVF [8,125–127]. Both SVF and ASCs have recently gained wide-range translational significance in regenerative medicine, but are fundamentally different in cell composition and immunophenotype characteristics [126,127]. The SVF is a heterogeneous cell population comprising cells of endothelial, hematopoietic, and pericytic origin, among others. Cells of hematopoietic origin include granulocytes (15%), monocytes (15%), lymphocytes (15%), and stem and progenitor cells (<0.1%) [94]. Three major populations of stem/progenitor cells in SVF closely associated with small blood vessels including endothelial progenitor cells (CD45–/CD31+/CD34+), pericytes (CD45–/CD31–/CD146+), and the supra-adventitial ASCs (CD45–/CD31–/CD146–/CD34+) have been described [127–129]. ASCs are a relatively more homogenous,

Table 2
Comparison between bone marrow derived (BM-MSCs) and adipose tissue derived stem cells (ASCs).

Type of cell	BM-MSCs	ASCs
Cell source	Bone marrow, usually from iliac, femur, tibia and sternum bones	Subcutaneous white adipose tissue from trunk and extremities
Harvesting technique	Bone marrow (trephine) biopsy and aspiration; serious complications such as pain, bleeding, infection and death exist but rare [117]	Direct excision during surgery, and Liposuction (e.g. Coleman technique); comparatively easier, safer, and considerably larger amounts of samples are accessible [244–247]
Yield and expansion (per unit of tissue substrate)	100–1000 MSCs, Easier isolation and <i>in vitro</i> expansion (3–5 weeks), Chromosomal instability with more passaging (Senescence)	Approx. 5000 MSCs [98,118–120], Higher number of population doublings [305], Have a greater capacity of clonogenicity - more elevated rate of colony-forming unit fibroblast [19], Better chromosomal stability with more passaging [121]
Cytometric characteristics	Applicable to the minimum criteria, i.e. presence of CD105, CD73, CD90 (≥95% positivity) and absence of CD45, CD34, CD14 or CD11b, CD79a or CD19 (≤2%) and cannot express HLA-DR [93], Expresses CD106 (VCAM-1) molecule which identifies a subpopulation of mesenchymal stem cells with unique immunomodulatory properties [306]	Not completely applicable to the minimum phenotype requirements, Phenotypically defined by absence of CD45, CD235a, CD31, CD106 and presence of CD73, CD90, CD10, CD26, CD49d, CD49e and CD146 surface markers [59], Specifically, express key extinguishing marker of isolation, CD34 [19,129,306].
Secretome function	Higher concentration of VEGF	Greater pro-angiogenic capacities, Appreciable functional properties regarding inhibition of inflammatory and immune responses [42]

adherent and culture expanded cell population containing both adipose derived stromal cells (ADSCs) and MSCs [8]. Most studies indicate that depending on the processing, ASCs represent up to 10% of the SVF [127,130].

ASCs like other MSCs, have been widely studied and increasingly becoming an MSC product of choice in clinical applications [79,95,131–133]. These cells exhibit excellent proliferation and differentiation ability into endodermal [134,135], mesodermal [136–138], and ectodermal cell lines [139]. Liposuction is a less sophisticated procedure with minor adverse effects and ethical restrictions (Table 2) [114,140]. ASCs can be isolated from adipose tissue using two approaches: Enzymatic and Non-enzymatic (Mechanical). Introduced by Zuk and colleagues [8], the enzymatic approach utilizes collagenase type II to digest adipose tissues, and has long been developed and optimized. Although popular, and considered the most useful and effective for clinical use, this procedure still requires standardization. Hence, the establishment of a standard protocol for isolation of ASCs that generates comparatively more viable stem cells (9.06×10^5 cells per 100 mL of adipose tissue with 99% cell vitality), in a shorter period of time with reduced risks of contamination [141]. The non-enzymatic approach is reportedly more economical, but less effective [105,142].

1.2.3.1.1. The unique morphological characteristics of ASCs. Due to the heterogeneous nature of ASCs, cells should be characterized each time before being used in clinical applications. Phenotypic validation is part of the safety evaluation that ensures that the isolated and expanded cell population is genuinely MSC phenotype. Immunophenotypic analysis should be performed following both the isolation and expansion phases, as part of the criteria for using the cells in clinical applications. ASCs are distinct in terms of surface-marker expression profile [143], but a unique single marker that characterizes these cells remains elusive. Therefore, during immunophenotyping the use of a multicolor identification panel of several cell surface markers is recommended. Additionally, a viability marker is also suggested to eliminate dead or apoptotic cells. The selected markers for classification of ASC populations may vary, as any variation in isolation and culture can influence their morphological characteristics [105,127,144]. In addition to the existing recommendations [93,94], ASCs should be positive for surface markers CD10, CD13, CD29, CD44, CD166, and negative for CD13. Also, there is a perceptible list of some indeterminate markers such as CD31, CD106, CD146. ASCs reportedly have moderate levels of CD34 expression, particularly during the early phase of cell culture, that decreases with continued cell division [145,146]. Multiple classes of CD34 antibodies exist that recognize unique immunogens, and the choice of CD34 antibody can substantially influence the signal intensity detected on a given cell population. Moreover, the histological analysis of adipose tissue has revealed that CD34-positive cells are primarily associated with vascular structures [125]. Although small numbers of these cells are probably CD31-positive capillary endothelial cells, a CD34+/CD31- cell population of pericytic origin may be derived from adipose tissue [147]. Original ASCs lack CD45, CD235a and CD31 markers, while cultured ASCs express CD73, CD90, CD105, and CD44, but are negative for CD45 and CD31.

To distinguish ASCs from BM-MSCs, the use of CD36 (fatty acid translocase) and CD106 (VCAM-1) markers has been proposed. In contrast to BM-MSCs, ASCs do not express CD106 but are moderately positive for CD36 [94,146,148,149]. Additional markers can further strengthen ASC characterization. For instance, Bourin and coworkers have suggested that CD10, CD26 (DPPIV), CD49d (VLA4), CD49e (VLA5), and CD146 (MCAM) can be included as additional positive markers, but with variable expression, depending on donor or culture passage. In contrast, low expression (<2%) levels of additional negative markers—CD3, CD11b (Mac-1), CD49f (VLA6),

and podocalyxin-like protein—can be observed. Nevertheless, it is likely that ASC populations, identified using basic surface antigens, will display heterogeneity for additional surface antigens [148].

1.2.3.1.2. Multiple factors for ASCs preference. A review article by Pablo and Majumdar [150] has highlighted reports on the distinctive, heterogeneous cellular composition of SVF contributing to better therapeutic outcomes observed in animal studies compared to ASCs. Moreover, SVF is reportedly much more easily acquired, without the influence of cell separation or culturing factors that could impact safety and other regulatory requirements. However, ASCs are preferable, firstly, for their suitability for both autologous and allogeneic treatment purposes. Allogeneic ASCs are generally preferred over other cell types due to their low immunogenicity as demonstrated by low expression of major histocompatibility complex (MHC) class II molecules, and T and B cell costimulatory molecules CD80, CD86, and CD40 in vitro [37,38,151]. Autologous ASCs are especially useful in applications where larger cell numbers are needed [152]. SVF is especially useful in soft tissue reconstruction and suitable for autologous treatments only because of its heterologous nature associated with increased chances of immunological rejection [153]. Secondly, culture expansion of ASCs improves cell homogeneity, precise identification and dosage, that are of paramount importance for obtaining the desired high reproducibility of clinical outcomes [97,154]. Thirdly, there are a number of peer-reviewed scientific reports demonstrating effects encouraging enough to merit the choice of ASCs-based therapy. For instance, (i) for various reasons the *in vivo* engraftment and survival of implanted SCs in regenerative therapy remain a challenge. ASCs however, can localize, survive and engraft in host tissue, and have the capacity of multi lineage differentiation within a new micro-environment [59], (ii) emerging evidence shows that MSC (including dead, or inactivated fragmented MSCs [155]) secretome is responsible for the cells' biological and pharmacological properties such as immunomodulation, immunosuppression, anti-inflammatory, anti-apoptosis, and angiogenesis [91,99–101]. Numerous studies [156–161] have demonstrated that the ASC secretome in particular has:

- higher levels of vascular endothelial growth factor (VEGF-D) mRNA and growth factors such as platelet-derived growth factor (PDGF), transforming growth factor (TGF- β), fibroblast growth factor (b-FGF) supporting angiogenesis and proliferation
- higher levels of pro-inflammatory cytokines such as interleukin (IL) –8, 1 β , 6, and 12, tissue necrosis factor (TNF- α), interferon gamma (IFN- γ) supporting recruitment and activation of innate and adaptive immunity cells, fibroblasts, and MSC
- anti-inflammatory cytokines such as; IL-10, 13 and Prostaglandin E2 mediating immunosuppression
- Chemokines such as IL-8, monocyte chemoattractant protein (MCP) MCP-1 (CCL-2), macrophage inflammatory protein (MIP) MIP-1 α (chemokine ligand CCL-3), MIP-1 β (CCL-4) promoting migration of innate and adaptive immunity cells, fibroblasts, and MSCs
- Adipokines such as Leptin, higher levels of insulin-like growth factor (IGF) IGF-1, adiponectin, steroid hormones, resistin, plasminogen activator-inhibitor 1 (PAI-1) for adipose tissue homeostasis
- Matrix proteins such as Collagen-1 for extracellular matrix (ECM) synthesis
- Matrix protease such as matrix metalloproteinase (MMP) MMP-1, MMP-2 for ECM remodeling and permitting cell transit and
- many other putative paracrine factors [162].

1.2.3.1.3. Overview of ASCs clinical application. The therapeutic utility of ASCs is based on autologous and allogeneic approaches.

ASCs in cell therapy can be applied in a single step as non-expanded cells or in vitro expanded cells. In vitro expansion helps in selecting a more homogenous cell population to meet standard criteria for MSC identification, and precise determination of cell dosage to ensure high reproducibility of the results [97], but it is costly and may present with concerns described later herein. The autologous ASCs approach involves adipose tissue harvesting from an individual, isolation and ex vivo expansion of the ASCs before transplanting them to the same individual. This often increases patient hospital stay or visits, and in peril of donor site morbidity, the most important being scars, infection and loss of function [163,164]. The allogeneic ASCs approach is based on the understanding that ASCs have a low immunogenicity in vitro, their secretome varies across different cell donors leading to highly variable outcomes, and are quickly available as an off-the-shelf product for immediate use [165]. Various reports have shown that allogeneic ASCs have the ability to elicit humoral and cellular immune responses *in vivo* particularly at sites of inflammation, and hence are not fully immune privileged [148,163,166–169]. However, a meta-analysis report of 82 preclinical studies has demonstrated that the approach is safe and as effective as autologous therapy [170]. Moreover, allotransplantation is very useful in especially the elderly and/or those patients affected by co-morbidities where autologous ASCs may have reduced regenerative and therapeutic potential [171–174]. Both approaches of ASCs-based therapy present some drawbacks and should to be considered on a case by case basis. Nonetheless, allogeneic ASCs seem to attract more clinical interest, as many patients can benefit from one or more donor cells having optimized characteristics and being selected for specific applications [165]. Recently, MSCs-based clinical trials mainly at phase I and II, have been conducted for a variety of human diseases with increasing utility of ASCs cell type. Chu et al. [175] in a recently published review have highlighted that the studies about ASCs human trials have been increasing year by year starting from 2007 and reaching its peak in 2015 with up to 187 clinical trials using adipose stem cells. Other reports [165] have indicated a total of 282 registered trials in late 2018, although 22 (8%) of them utilized SVF and only 13 (5%) had progressed to advanced phases.

The clinical utilization of ASC-based therapy is mostly in medical and surgical conditions including aesthetic, orthopedic, immune system, cardiovascular, craniomaxillofacial, skin and connective tissue, nervous system, nutritional and metabolic diseases. Clinical application details regarding some of the registered clinical studies

of ASCs for different diseases including Osteoarthritis, Ischemic heart disease, Critical Limb Ischemia, Amyotrophic Lateral Sclerosis, Multiple System Atrophy, and Spinal cord Injury are summarized in Table 3 [176–178]. The application of ASCs in aesthetic surgery is common in trials involving repair of soft tissue defects [179]. The fat grafting procedure has long been employed to repair soft tissue defects. However, it presents with substantially variable successes in different surgical hands, and significant complications such as loss of volume, fibrosis, and necrosis. Therefore, ASCs are used to take advantage of their angiogenic, survival enhancement, proliferation, and differentiation properties for the improvement of graft viability. Pioneering work involving the use of SVF in combination with intact lipoaspirate of tissue in breast reconstruction or augmentation had positive outcomes such as improved volume retention, no evidence of fibrosis or adhesions, but also a few minor complications such as cyst formation or microcalcifications [126,180–182]. Other reports have indicated significant improvement in the healing of post mastectomy radiated skin and dermis complications with both SVF and ASCs [183].

Although notable success in MSCs efficacy and safety has been published [184], after almost two decades of large investments, cell therapy for CVD, particularly in heart muscle regeneration using BM-MSCs and cardiac stem cells remain elusive [185,186]. Further rigorous studies on earlier established strategies to trigger heart repair and regeneration such as use of human heart progenitors and cardiomyocytes derived from pluripotent stem cells, tissue engineering of heart patches, paracrine-factor therapeutics with proteins, and others are recommended [187–189]. Such efforts could benefit from the use of ASCs and/or ASC-derived cardiomyocytes. For example, ADSCs have been used in a phase II (MyStromalCell) trial to treat patients with chronic ischemic heart disease. This was the first single-center, double-blind, randomized, and placebo-controlled study of intramyocardial injections of autologous adipose-derived MSCs in patients with chronic ischemic heart disease and refractory angina but preserved ejection fraction [177,190]. The MSCs were obtained from abdominal adipose tissue, culture-expanded in vitro and stimulated with VEGF-A a week before treatment. Six-month follow-up results demonstrated safety, and a significant increase in exercise capacity in patients treated with MSCs but not with placebo.

The use of ASCs for regenerative therapy directly depends on efficient adoptive transfer of the critical therapeutic effects in animal models including inter alia, proliferation, potency, homing/

Table 3
Clinical trials of adipose derived stem cells applications.

Disease	Clinical trials	Routine treatment	Effect of ASCs therapy	Autologous or Heterologous	Ref.
Knee osteoarthritis	Phase I	Intra-articular injection	Decrease pain and improve WOMUA index	Autologous	[182]
Degenerative disc disease	Yes	Injection	Decrease low back pain	Autologous	NCT02097862
Hip osteoarthritis	Yes	Percutaneous injections	Regenerate cartilage-like tissue	ASCs	[183]
Heart failure	Phase II	Intramyocardial injection		Heterologous	NCT0267316
Ischemic heart disease	Phase I/II	Intramyocardial injection	Increase myocardial perfusion	Autologous	[184]
Ischemic cardiomyopathy	Phase I	Intravenous injection	Angiogenic effect	Autologous	NCT00426868
Critical limb ischemia	Phase I/II	Intramuscular injection	Angiogenic effect	Autologous	NCT01211028
Chronic myocardial ischemia	Phase I/II	Intramyocardial injection	Angiogenic effect	Heterologous	NCT01556022
Ischemic stroke	Phase II	Intravenous injection	Angiogenic effect	Heterologous	NCT01678534
Stroke	Phase II/III	Intravenous infusion	Angiogenic effect	Heterologous	NCT02849613
Amyotrophic lateral sclerosis	Phase I	Intravenous injection	Safety Improvement of ALS function, FVC	ASCs	NCT02492516
Multiple system atrophy	Phase I	Intrathecal injections	Safety	Autologous	NCT02315027 ^a
	Phase I/II	intrathecally via lumbar puncture	Safety at high dose	Autologous	[185,186]
Spinal cord injury	Phase I/II	Intrathecal transplantation	Recover ASIA and sensory score	Autologous	[187]
Traumatic brain injury	Phase I/II	Injection	Safety Benefits	Autologous	NCT02959294 ^a

WOMUA index, Western Ontario and McMaster Universities Arthritis Index; ALS, amyotrophic lateral sclerosis; FVC, Forced Vital Capacity; ASIA, American Spinal Injury Association.

^a Clinical trials are processing.

migration, and paracrine actions [191–193]. Effective human clinical translations require careful attention to factors that affect performance as well as safety of ASCs. Numerous publications [165] have highlighted the impact of various factors on the function of ASCs including age, sex, body mass index (BMI), adipose tissue origin, donor comorbidity states such as diabetes, cell biological and culture factors. Highlighted in a recent review [127], the ability of stem cells to renew appears to decrease with aging and is associated with down-regulation of SIRT-1 (silent information regulator 1) by micro-RNAs; miR-486-5p has been proposed to play a role in ASCs replicative senescence [194,195]. Aging is associated with down-regulation of genes responsible for maintenance of genomic integrity and chromatin remodeling, leading to functional attenuation and risk of neoplastic transformations [196]. Moreover, the influence of age on tri-lineage differentiation potential of MSC has been demonstrated to negatively affect osteogenic potential and enhance adipogenic potential [197], suggesting differential growth factor expression in senescence, whereby there is a reduction in BMP2/4 and upregulation in TGF- β expression [198]. Also, aged MSC are known to express higher levels of pro-inflammatory cytokines, in the absence of cytokine and chemokine receptors, impairing their ability to respond to injury [199]. Distinct developmental origins of adipose tissue potentially affect cell expansion, differentiation, and therapeutics. It has been demonstrated that different visceral fat depots are remarkably heterogeneous, and gene expression profiles and differentiation capabilities differ significantly between ASCs derived from different fat depots. Moreover, the depth of adipose tissue harvest appears to be critical on ASC proliferation and adipogenic potential, as ASCs from subcutaneous adipose tissue have increased proliferation and adipogenic capacities compared to ASCs of visceral origin [165]. Finally, as mentioned earlier conditions including but not limited to cell isolation and culture can affect ASC proliferation, differentiation, and paracrine function [200–202].

Nevertheless, some reports have highlighted safety concerns related to the use of *in vitro* expanded MSCs [203–206]. The expansion of cells outside their natural environment is thought to increase risk for genomic instability or altered differentiation potential, and may be associated with significant adverse effects such as tumors, teratomas and severe immune reactions, perhaps due to lack of counter immune surveillance and the influence of different culture induction factors. Regarding genomic stability, it has been demonstrated that cultured ASCs are stable in long-term cultures after multiple cell doublings, even under xenofree conditions [207–210]. However, there is controversy when it comes to the risk of neoplastic transformations, which requires further studies. The cytokine features of promoting angiogenesis, cell migration, proliferation, renewal, epithelial trans-differentiation and immunomodulation for MSCs are reportedly potentially tumorigenic. MSCs can be attracted to sites of tissue injury as well as tumor micro-environments, and are able to differentiate into myofibroblasts known to promote tumor growth; such features are key contributors to tumor aggression and invasiveness in breast lipofilling [211,212]. BM-MSCs have been shown to increase tumorigenicity and invasiveness of breast cancer cells by inducing *de novo* expression of CCL5, a chemokine that acts in a paracrine manner to increase cell migration (*in vitro*) and extravascular translocation (*in vivo*) [206]. Similarly, induced *de novo* expression of the chemokine CXCL-12 (SDF-1) has been reported in ASCs, which increased tumorigenicity and invasiveness that were reversed by inhibition of the corresponding receptor [213]. Among the additional reports supporting the possibility of malignant transformations are the popular report on sarcoma formation in immuno-deficient mice following the injection of *in vitro* post-senescence transformed ASCs [214] and that on the use of fetal

calf serum in cell culture presenting a risk for prion exposure [215]. The former was retracted because, later it was shown that the cells used in the transformation studies were cross-contaminated by cancerous cells that initially grew slowly in the presence of human MSCs [216], and the latter remains debatable. However, ASC and ASC-conditioned supernatant have been shown to induce necrosis in a range of tumor cell lines *in vitro* and *in vivo* [217]. A recent study designed to evaluate whether fat engraftment offers a supportive environment for tumor growth concluded, emphatically, has shown that it does not and may even be suppressive [218]. This conclusion appears to be supported by reviews of clinical studies of fat grafting for oncological breast reconstruction [219,220]. Other safety concerns arguably include exhibition of highly procoagulant activity and even lethal effects upon infusion of ASCs in preclinical models [221–223], and cases of peripheral microthrombosis, pulmonary embolisms, and even suspected cases of death in patients receiving ASC intravascular infusions [224–226].

Nonetheless, MSC products are generally considered safe in the clinic [227]; the international federation for adipose therapeutics and science (IFATS) has not yet reported ASCs based clinical studies associated with increased risk for adverse events/infusion toxicity [228]. Moreover, in a recent dose-escalation study, intravenous infusions of ASCs from healthy donors were well tolerated by humans up to 4×10^6 cells/kg body weight [229]. Remarkably, currently there are efforts to encourage the use of regenerative therapy including the application of ASCs as an MSC product in different countries across the world. For instance; the United States of America, the European Union, and Japan have initiated expedited approval processes aimed at giving patients better access to innovative drugs and regenerative medicine products albeit still subject to changes in the future [230,231]. Perhaps through clinical experiences, several hurdles or limitations can quickly be identified and solutions forged in a relatively shorter time frame.

2. Research involving the use of culture expanded ASCs

2.1. Investigating the therapeutic impact of ASCs in aesthetic surgery

The University of the Ryukyus hospital is the leading institution in advancing stem cell research in Okinawa, Japan. The institution actively implements basic, translational, and clinical research on ASCs, for a substantial contribution to the promotion of regenerative medicine through various studies at its Regenerative Medicine Center and the advanced medical care specialties. To conduct research in accordance with local and international standards, the university hospital launched a Cell Processing Facility (CPF) in March 2015. The CPF was licensed for Good Laboratory Practice (GLP) and Good Manufacturing Practices (cGMP) according to guidelines set by the regulatory framework of Pharmaceuticals and Medical Devices Agency (PMDA) and the Ministry of Health, Labor and Welfare (MHLW) [232,233]. In addition, the CPF is engaged in the highly skilled technical and clinical specialties of processing and applying viable cell therapies. In early 2016, the hospital department of plastic and reconstructive surgery took advantage of the existing vibrant collaborations and the GMP facility, and initiated a clinical trial with culture expanded ASCs to recontour depressed scars for the first time in Japan. Depressed scars of different origin can lead to cosmetic and psychosocial challenges, as well as the possibility of impeding normal body function. Several surgical approaches to treat such lesions have been described, but they are only adopted in selected cases and may achieve suboptimal results [234]. The approach of combining fat grafting with *ex-vivo* cultured cells is still under investigation. Published work by Coleman in the mid-1990s on treating depressed scars with fat grafting

[235–238], has dramatically changed transplantation techniques over the last two decades to strategies that, if properly executed, could have a high regenerative potential for both simple volume replacement as well as functional enhancement of recipient tissues [239]. In 2006, Matsumoto et al. [126] provided evidence to support a novel method (Cell-Assisted Lipotransfer-CAL) to boost efficacy and reduce adverse effects of lipoinjection. Also, Rigotti et al. [183] have reported high therapeutic effects when degenerative, chronic lesions due to oncologic radiation were treated with lipoaspirates containing ASCs. Moreover, studies on condensation of ASCs led to the increased application of fat grafting in the revitalization of stem cell-depleted tissues [240–242]. In addition to the excellent regenerative functions [59,243], it has been suggested that the purity of ASCs is critical for their therapeutic outcomes [244], and development of biotechnological techniques have greatly improved the use of highly purified ASCs [245,246]. As a result, ASCs might play a primary role in the regenerative medicine of the 21st century, provided that risk factors related to manipulation, cryopreservation, concentration, and route of administration are controlled and standardized [244]. Thus, with the available resources the department decided to actively investigate the application of fat grafting in combination with culture expanded ASCs in cosmetic surgery.

2.1.1. ASCs clinical study implementation

The ASCs clinical trial at University of the Ryukyus hospital dubbed “Clinical study of treatment for depressed lesions using cultured adipose derived stem cells,” was an investigator-driven, open-label, non-randomized, uncontrolled, phase I/II study. It was conducted in accordance with the 2014 enacted Act on the Safety of Regenerative Medicine (ASRM) [232,233]. Thus, the department was granted ASRM Class II approval in line with treatments using cells from a patient but performing a different function. Moreover, the trial was in compliance with the study protocol, the International Conference on Harmonization’s Good Clinical Practice standards, and the Declaration of Helsinki. Approvals for the protocol, subject information and informed consent forms were obtained from the institutional review board (IRB) of Ryukyu University (No. 857). The trial is registered under one identifier at UMIN-CTR Clinical Trial (<https://www.umin.ac.jp/ctr/>) with unique ID number; UMIN000020530.

Based on the underlying treatment concept and on the promising data taken from preclinical and clinical studies regarding the use of ASCs in treating a wide range of other conditions, for evaluation of safety and probable efficacy a maximum number of 11 patients was targeted for this clinical trial. Participation was restricted to 10 years old and above male or female patients with (a) congenital or acquired depressed lesion for which fat grafting was advised, such as breast deficit after breast cancer, traumatic tissue defects, hemifacial atrophy, facial muscle atrophy after facial palsy, enophthalmos due to anophthalmia, funnel chest, and others, (b) depressed lesions undergoing secondary revision surgery with fat grafting, (c) in case of secondary revision surgery, at least three months should have passed after first surgery, (d) willingness to provide informed consent, (e) history of at least 10 years of no serial illnesses, (f) willingness to live in Okinawa prefecture at least 6 months following treatment, and finally (g) potentially eligible patients participating in another study about developing an extraction system of safe and good quality adipose derived stem cells were considered. However, patients who were pregnant, at risk of recurrence or metastasis of a malignant tumor, under anti-cancer agent or immunosuppressive agent treatment, or those who had local infection in or near the depressed lesion were excluded.

The trial design was interventional, and followed standard fat grafting techniques commonly performed in three stages:

harvesting of adipose tissue from a suitable donor site, processing of the lipoaspirate to eliminate cellular debris, acellular oil and excess of infiltrated solution, reinjection of the purified adipose tissue [242]. Specifically, for this trial a small amount of fat was aseptically transferred to the CPF for the isolation and expansion of ASCs prior to administration into the depressed lesions in combination with free fat.

Adipose tissue was carefully harvested and processed with a procedure that aimed at minimizing massive destruction of adipocytes and maximizing yield of ASCs [247–250]. The technical details of harvesting, GMP processing and transplantation of the cultured ASCs will be published in the final scientific reports of the trial. Anticipated outcomes of the trial include improved graft survival rate, reduced calcification, and cyst formation as well as incidence, severity, and duration of adverse events following 1 week, and 1, 3, 6 months after treatment.

Preliminary data are available for 5 patients with depressed facial scars caused by complications ranging from cancer treatment to Parry-Romberg syndrome. The first trial subject was a case of malar deformity following surgical management of maxillary sinus carcinoma (Fig. 1). All the patients were treated and followed up according to the study protocol. Briefly, Informed consent to treatment and publication of outcomes was obtained in writing from the patients prior to any procedures. Evaluation of clinical improvement was based on patient interviews and standard photographs taken before, 1 week, and 1, 3, and 6 months after treatment with cultured ASCs. In addition, computed tomography scans were taken to assess the volumetric change before and 6 months after treatment. Results suggested that the patients’ depressed scars were recontoured with satisfactory results, and no adverse events were reported even after two and half years, a period outside the study follow up schedule.

Such promising data obtained during this phase I/II trial represent the first milestone in the clinical evaluation of the concept of repairing soft tissue defects with fat grafts combined with culture expanded ASCs in Japan. Fat grafts have an important role in the treatment of depressed or altered scars and other surgical problems [242]. So far, the trial experiences are in agreement with previous reports [105,141,251]. The department faces similar challenges, particularly related to the high cost of clinical grade ASCs production and sustainability of the GMP facility, the long time required to process and prepare cells for transplantation (3–5 weeks), and the variability in the autologous ASCs product and use of biological reagents, which are likely to influence clinical outcomes.

2.2. Approaches to enhance the regenerative capacity of ASCs

2.2.1. The tissue engineering approaches

Despite developments in different aspects of clinical practice, there are still shortcomings related to availability of donor tissues that require novel approaches to overcome. Tissue engineering (TE) is a highly interdisciplinary research field driven by the goal to restore, replace, or regenerate defective tissues [252–254]. As this is in tandem with the objectives of the advanced medical services, the university hospital regards TE a concept of paramount significance. Hence, the clinical application of ASCs with this concept is under intense investigation and various approaches have been proposed in the different specialties including orthopedic, plastic and maxillofacial surgeries [255].

Historically, Atala and co-workers [256] were the first to report the use of tissue-engineered constructs in patients. Between 2008 and 2014, a range of engineered tissues including the trachea [257,258], urethra [259], and nasal cartilage [260] were reported, arguably with questionable successes [261]. Remarkably however, TE has overcome a number of challenges that had hindered its

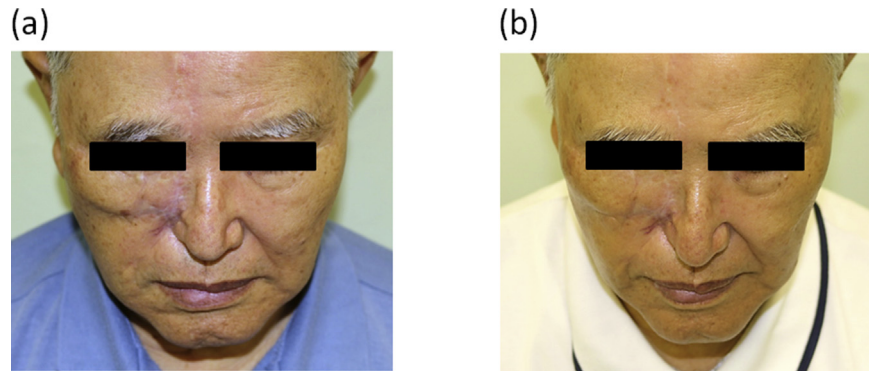


Fig. 1. Pre- and Post-operative images of the first clinical trial subject. (a) Pre-operative image showing malar deformity following treatment of Maxillary cancer. (b) Two and half years of post-operative follow up image shows a near normal contour line of the patient's face.

clinical application in the past [253,262], although some of them such as vascularization of larger volume tissues [263,264], and risk of tumorigenesis [265] remain of concern. Tremendous progress toward improving the methodology for TE is notable through areas of (i) discovery of methods to generate functional cells such as the iPS and adult stem cells from different sources, (ii) substrate stiffness establishment as a modulator of stem cell differentiation leading to new ways of controlling cell phenotypes using physical cues, (iii) advanced chemistries that enable more efficient and versatile biomaterial conjugations, for precise patterning of biomolecules and biomaterials, (iv) refinement of delivery mechanisms that enable biochemical cues such as growth factors and cytokines to be presented with improved bioavailability and bioactivity, (v) understanding of the interaction between foreign bodies and the body's immune surveillance system, that promote rational design of biomaterials to mitigate inflammatory responses, (vi) development of new biomaterials and scaffolds that lead to fabrication of better biomimetic tissues, (vii) advances in bio-fabrication technologies including programmed self-assembly and three-dimensional (3D) bioprinting, which allow generation of complex biological structures with integrated vasculature and multiple cell or extracellular matrix types at high spatial resolution [266]. It has been suggested that advances in these areas could potentially eliminate the need for donor specific sites and their morbidity, reduce hospital stay and associated costs in the field of plastic surgery [267].

The fundamental requirements for providing durability of tissue-engineered constructs include a cell source to synthesize new tissue matrix, a scaffold, biomolecules and a microenvironment to provide trophic cues that guide proliferation and differentiation [268]. TE uses tissue-derived cells that may be stem cells or not, but currently there is no consensus on the ideal source for such purposes. Thus, a thorough understanding of the advantages and disadvantages of each cell type is crucial for the selection of the appropriate cells and the optimal culture conditions in order to engineer specific tissue types [267]. The technological approaches of TE can be principally categorized into two conventional strategies; scaffold-based and scaffold-free. Some reports suggest a third strategy which integrates the advantages of the two traditional approaches [254], but this is beyond the scope of this review.

2.2.1.1. Scaffold free-cell sheet technology. Scaffold free is a bottom-up strategy employing cell sheets, spheroids, or tissue strands as building blocks. It relies on the inherent ability of these building blocks to fuse and form larger constructs [254]. Cell sheets are 3-D cell structures consisting of multiple layers of confluent cells arising from high-density cell seeding that are transplantable

without an additional carrier [269]. There are several methods for culturing cell sheets, including high-density seeding or incubation with magnetic nanoparticles-liposomes [270,271]. Also, different culture surfaces are used to form cell sheets and assist cell sheet detachment. Besides conventional tissue culture polystyrene dishes with or without a special coating, temperature-responsive culture dishes are being used [272]. Temperature responsive culture dishes allow cell sheets to be easily harvested by changing the dish temperature. These temperature responsive culture dishes are coated with a polymer named poly (N-isopropylacrylamide) (PIPAAm), which has extraordinary properties. At 37 °C the surface is slightly hydrophobic allowing cell attachment and proliferation. When the temperature is lowered to 32 °C or below the surface become hydrophilic and the polymer expands causing spontaneous detachment of cell sheets [273]. This technique leads to less cell damage compared to traditional methods of cell detachment that utilize enzymatic digestion or mechanical peeling which may disrupt cell structures. Moreover, temperature-responsive culture dishes preserve cell surface adhesion proteins and the extracellular matrix (ECM) connecting the cells, which may facilitate cell sheet attachment to tissues [274].

Cell sheet technology is a promising cell-based treatment strategy that can be used in both tissue engineering and regenerative medicine [275,276]. Cell sheets can be stacked to create a 3-D tissue in culture. Different cell types can be combined in a cell sheet to enhance tissue repair and revascularization. For example, endothelial cells can be combined with different tissue cell types to promote angiogenesis of cardiac tissue [277], skin [271] or oral mucosa [278] repair. MSC sheets have been studied in several disease models in order to enhance healing of injured tissue, such as cardiac muscle [271,275,279], skin [280], bone [281] and cartilage [282]. The downside of this approach is that the inferior mechanical properties of individual building blocks, lead to possible cell damage during their manipulation, and the immobilization phase necessary for the initial fusion of the building blocks and deposition of ECM to obtain a cohesive construct is long [271,283]. ASC have shown increased angiogenesis and tissue healing when injected or delivered via a scaffold. However, injection provides inefficient delivery of cells and scaffolds are accompanied by the risk of infection. Delivering ASC in the form of a cell sheet offers a unique delivery method that may best take advantage of these cells. There has been a limited number of studies examining the used cell sheets composed of ASC, for instance, in the repair of scarred myocardium in rats [279,284]. However, better understanding of ASC sheet characteristics, therapeutic potential and application areas are needed in order to optimize ASC cell sheet as a tissue repair strategy.

2.2.1.2. Scaffold-based cell sheet technology. The scaffold-based approach relies on the use of appropriate ‘templates’ supporting living cell attachment, proliferation, and subsequent formation of 3D tissue [254]. Scaffolds are generally classified as biological (organic) or synthetic (inorganic). Desirably, scaffolds should be biomimetic [285] biodegradable [286], have appropriate mechanical strength and optimal micropores enabling vascularization and allowing metabolic needs to be met. They should also be biocompatible [287], non-immunogenic [288], and versatile with regard to manufacturing methods, functionalization potential [289], and 3D control of microarchitecture. It is important to understand that the biophysiochemical 3D environment is crucial for tissue engineering. Cells not only require a scaffold for structural and biological support but also require an environment that provides the correct combination of growth supplements, differentiation signals, perfusion of nutrients, gaseous/waste exchange, pH regulation, and mechanical forces.

2.2.2. Investigations with scaffold based cell sheet technology

Investigations to improve the differentiation potential of ASCs towards target tissue cells including inter alia cardiomyocytes, chondrocytes and tenocytes are required. For instance, several novel approaches are now sought to improve heart muscle regeneration in the field of cardiovascular cell therapy [185], and ASC transdifferentiation could be a viable approach. Therefore, the University of the Ryukyus hospital department of plastic and reconstructive surgery in collaboration with the Regenerative Medicine Center, aims to decipher critical factors affecting ASC transdifferentiation. A deeper understanding of how different culturing conditions and mechanical forces impact the regenerative abilities of ASCs using the scaffold-based cell sheet technology is being actively pursued.

2.2.2.1. Acquisition of ASCs for the research. After lipoaspirate collection from aesthetic surgery patients at the University of the Ryukyus hospital, the adipose tissue is separated from the tumescent solution, oil, serum, cell debris, and blood by centrifugation. The adipose tissue was mainly used in graft transplantation, and prior to current regenerative therapy interests, all excess tissue with the rest of supernatant debris were discarded. However, currently with the informed consent of patients, part of the excess tissue is quickly transferred in cooler boxes at 2–8 °C, under aseptic conditions to the basic research facilities for isolation, expansion, characterization, and differentiation as well as pre-clinical studies of the ASCs. The isolation of ASCs from freshly harvested adipose tissue is based on well-established protocols [290], but with slight modifications. The tissue is transferred to appropriate sterile centrifuge tubes, labeled, weighed, and washed in wash buffer. Thereafter, through a repeated process the tissue is completely digested by equal volumes of 0.25% trypsin and 0.1% of type I collagenase, minced and thoroughly mixed under incubation at 37 °C in 5% CO₂ within an hour. The process of tissue digestion is stopped by stromal medium and through a series of strainings and centrifugations, SVF is separated from free lipids and mature adipocytes. After several washing steps and an optional erythrocyte lysis, the SVF cells are resuspended in growth medium such as high glucose Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotic–mycotic solution at 37 °C with 5% CO₂ and cultured for 24 h to produce the plastic adherent ASCs. Growth medium is changed every 2–3 days until the ASCs reach an appropriate confluence suitable for further studies. To keep cells at low density, preventing cell death and spontaneous differentiation, cultures are passaged using 0.25% trypsin/ethylene diamine tetra acetic acid on reaching 80% confluence. Because the differentiation potential and mechanical

properties of MSCs decline with extended cell passaging, its recommended to utilize cells between the second and fifth passages [291].

ASCs are verified according to the ISCT and IFATS criteria including use of flow cytometry to quantify surface marker expression, and the ability to differentiate into osteoblasts, adipocytes, and chondroblasts under in vitro conditions [93,94]. In addition, relevant assays to monitor cell viability and proliferation are performed as previously described [292].

2.2.2.2. Exploring factors that influence the regenerative potential of ASCs. Investigations into the impact of different factors on ASC transdifferentiation are exemplified in this review by considering cardiomyocytes differentiation. There are various cardiomyocyte differentiation techniques based on different cell induction approaches, each one having its own advantages and disadvantages [138,293–299]. The investigation of factors related to culture conditions involves differentiation of ASCs towards cardiomyocytes by adding various supplements and growth factors to the basic medium. For these ongoing studies, ASCs are differentiated to cardiomyocytes through an initial 24 h induction phase using 5-aza-2-deoxycytidine in DMEM. Here, DMEM is supplemented with 15% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 1% Insulin-Transferrin-Selenium (ITS) + premix (6.25 µg/ml of human recombinant insulin and transferrin, 6.25 ng/ml of selenous acid, 1.25 mg/ml of bovine serum albumin, and 5.35 µg/ml of linoleic acid). The induced culture is maintained by changing the supplemented DMEM every third day for 4 weeks.

For the evaluation of factors related to mechanical forces, the concept of scaffold and 3-Dimensional culturing is explored. In the recent past, three-dimensional constructs have been developed to foster cell – cell communication and overcome growth inhibition by mimicking the physiological milieu. Also, scaffolds covered with different chemotactic agents, as well as matrices of varying stiffness values have been designed to achieve directional migration and stable cell cultures. In line with developing this technology, we have recently published work on modulation of surface stiffness and cell patterning [300]. Here, a new technology was developed to selectively produce areas of high and low surface Young’s modulus on biomedical polymer films using micropatterns. When NIH3T3 fibroblasts were cultured on micropattern-supported films, they preferentially accumulated in regions with high Young’s moduli, suggesting that the technology can regulate regions of high and low surface Young’s modulus on a cellular scaffold with high planar resolution and direct cellular patterning.

Non-woven fiber scaffold sheets are used to investigate the influence of mechanical stress over cell differentiation. Before ASCs seeding, the scaffold sheets are prepared; they are placed in well-plates with a supporting O-ring on top for stabilization, and sterilized before adding growth media. Additionally, any existing air bubbles in each well are degassed and the wetted sheets are incubated overnight at 37 °C. The ASCs at passages between 2 and 5 are seeded onto the scaffold sheets at a determined cell density and cultured in standard growth media until they reach 60–70% confluency before subjecting them to the 5-aza-2-deoxycytidine induction phase. The culture medium is replaced every third day after induction of cell differentiation.

The induction of cell differentiation under mechanical stress is an innovative concept in artificial tissue generation. It has been established that the cytoskeleton interprets and responds differentially to mechanical forces from the microenvironment [301]. In this context, cellular actin filaments have been shown to be a key initial regulator of cell morphology in response to extracellular mechanical forces [301]. Although preliminary results show high potential and promising future for mechanical cardiomyocyte

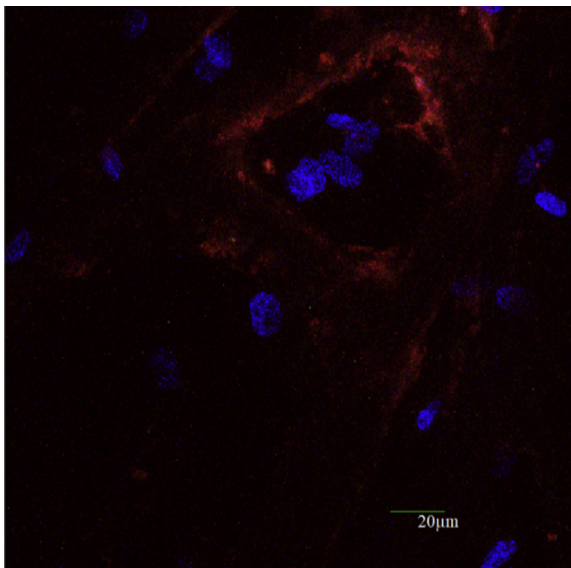


Fig. 2. Anti-CTnT antibody immunostaining for the detection of ASCs differentiated cardiomyocytes on a Non-woven scaffold.

differentiation in tissue engineering, the underlying molecular mechanisms are yet to be deciphered.

2.3. Observation of ASCs cardiomyocyte differentiation

Numerous methods are being applied to monitor and evaluate the process of cardiomyocyte differentiation. These methods range from lineage-specific immunological assays to the direct detection of cardiomyocyte-specific protein expression such as cardiac-specific transcription factors, including GATA4, homeobox protein NKX2.5, myosin regulatory light chain 2, ventricular/cardiac muscle isoform (MLC-2), myosin regulatory light chain 2, atrial isoform (MLC2a), cardiac troponin T (cTnT), troponin I (cTnI) and others (Fig. 2). Additionally, real-time polymerase chain reaction (PCR), Western blot analysis, and microarray analysis techniques are being performed. Lipoaspiration, a common procedure in aesthetic surgery, provides a significant amount of adipose tissue for obtaining ASCs, which represents a very promising resource for further research in tissue engineering compared with other mesenchymal stem cells. Human ASCs have good potential to differentiate into various tissue target cells including cardiomyocytes under varied induction stimuli. However, an ideal technique that would enhance ASC differentiation and *in vivo* regenerative capacity remains elusive owing to numerous challenges such as mode of cell delivery, cell homing efficiency, engraftment, clinical safety, and rejection of allotransplants.

3. Conclusion and further perspectives

Cell-based therapies involving stem cell products such as the MSCs are indeed promising approaches to the treatment of disorders for which current medical and surgical solutions are obscure [184]. Because multiple factors negatively affect stem cells and/or progenitor cells, characterization of function is important to guide more efficacious cell-based therapeutic applications. The magnitude of impact and strategies to ameliorate the regenerative potential of cells remain elusive. For example, a narrative review of MSC function in CVD [302] provided a systematic evaluation of a total of 41 studies examining CVD-related MSC (dys)function. Their findings revealed that CVD affects MSC characteristics and

regenerative potential but many of these studies presented conflicting results. Remarkably though, this and many other studies in different treatment fields have contributed enormously to the comprehension of complex biological processes underlying regeneration regardless. This has enabled the current understanding that the classical concept of regeneration where an insulted tissue or organ can be repaired by the administration and engraftment of large numbers of cells – stem cells or not – is inadequate. Instead, as reported previously, regeneration should be considered as a global and balanced process involving the entirety of target tissue structures in addition to the cell types [303]. Furthermore, to achieve the ultimate goal of building functional tissues or organs, the following priority areas of research are suggested: in-depth understanding of cell proliferation processes and their control mechanisms, development of means to identify and track cell subpopulation(s) capable of division in the insulted target tissue or organ based on ploidy and other characteristics, identification of markers of cell proliferation rather than of cell cycle activity, analysis of transcriptional pathways underlying target tissue cell dedifferentiation, replication, migration, and maturation [303].

The practical advantage of adipose tissue derived MSCs in clinical practice is their primary source – the adipose tissue being abundant and easy to obtain with minimal adverse effects. However, compared to ESCs and iPS cells, the multipotency of ASCs is limited. Moreover, their differentiation potential can be influenced by multiple factors such as anatomic location of fat, the donor's gender, age and comorbidity, and the key molecular mechanisms of proliferation and differentiation remain unraveled [304]. With promising data from the current research at the university of the Ryukyus hospital, and the guidance from related experiences elsewhere, carefully designed, and enriching studies are anticipated. Following completion of the assessment of safety and effectiveness of site-specific autologous ASCs instillations, the university hospital department of plastic and reconstructive surgery plans to conduct clinical evaluation of allogeneic ASCs in treating various diseases. Moreover, in the near future, pre-clinical studies will be considered as well, to assess the therapeutic impact of site-specific administration of combined cell types: regenerative and bonafide differentiated cells, using the scaffold based cell sheet technology.

Declaration of Competing Interest

The authors have no conflicts of interest to declare for this work.

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