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Review

A comprehensive understanding of UA-ADRCs (uncultured, autologous, fresh, unmodified, adipose derived regenerative cells, isolated at point of care) in regenerative medicine

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Abstract: It has become practically impossible to survey the literature on cells derived from adipose tissue with the aim to apply them in regenerative medicine. The aim of this review is to provide a jump start to understanding the potential of UA-ADRCs (uncultured, unmodified, fresh, autologous adipose derived regenerative cells isolated at the point of care) in regenerative medicine. We show that serious and adequate clinical research demonstrates that tissue regeneration with UA-ADRCs is safe and effective. ADRCs are neither 'fat stem cells' nor could they exclusively be isolated from adipose tissue, as ADRCs contain the same adult (depending on the definition) pluripotent or multipotent stem cells that are ubiquitously present in the walls of small blood vessels. Of note, the specific isolation procedure used has significant impact on the number and viability of the cells and hence on safety and efficacy of UA-ADRCs. Furthermore, there is no need to further separate adipose-derived stem cells (ASCs) from ADRCs if the latter were adequately isolated from adipose tissue. Most importantly, UA-ADRCs have the physiological capacity to adequately regenerate tissue without need for manipulating, stimulating and/or (genetically) reprogramming the cells for this purpose. Tissue regeneration with UA-ADRCs fulfills the criteria of homologous use.

Keywords: adipose derived regenerative cells; ADRCs, efficacy; point of care treatment; randomized controlled trials; safety; stem cells; stromal vascular fraction.

1. Introduction: what are UA-ADRCs and how are they used in regenerative medicine?

The literature on cells derived from adipose tissue with the aim to apply them in regenerative medicine has become practically impossible to survey, even for experts. A search in PubMed on "adipose derived stem cells" on 22 February 2020 yielded over 10,000 citations, among them approximately 1000 reviews. Furthermore, there is still disagreement about the term "pluripotent stem cell", "multipotent stem cell", stromal vascular fraction (SVF), adipose derived regenerative cells (ADRCs) and adipose derived stem cells (ASCs) in the literature. For example, a very recent study defined microvascular pericytes as true pluripotent adult stem cells with the ability to produce structures typical for the three primitive germ layers (ectoderm, mesoderm and endoderm) [1]. This is in contrast to, e.g., a definition of pluripotent stem cells provided by the U.S. Library of Congress as embryonic stem cells with the ability to become any type of cell in the body (nerve, muscle, blood, etc.), in contrast to multipotent stem cells that develop from pluripotent stem cells as the embryo grows, with the ability to develop specific types of cells (terminally differentiated cells) [2]. According to the latter definition microvascular pericytes could not be considered pluripotent as long as it would not have been demonstrated that these cells have the ability to become any type of cell in the body. Ultimately, this would require to develop a full body from a few microvascular pericytes (like the full body is developed from a few embyronic cells), which appears impossible. Of note, the latter would also apply to so-called induced pluripotent stem cells (iPSCs). According to a definition provided by the U.S. National Institutes of Health (NIH) iPSCs are adult cells that have been genetically reprogrammed to an embryonic stem cell-like state by being forced to express genes and factors important for maintaining the defining properties of embryonic stem cells [3]. In the view of the NIH mouse iPSCs demonstrate important characteristics of pluripotent stem cells, including the expression of stem cell markers, formation of tumors containing cells from all three germ layers, and the ability to contribute to many different tissues when injected into mouse embryos at a very early stage in development [3]. However, according to the aforementioned definition provided by the U.S. Library of Congress iPSCs should strictly speaking not be called pluripotent. Coming back to the pluripotent or multipotent (depending on the definition) cells in the walls of small blood vessels, the fact that it has been demonstrated that these cells are different from pericytes [4] (as stated in [1]), could make the confusion complete.

In our opinion the only way out of this confusion is to comprehensively provide in any study describing the use of cells in regenerative medicine a detailed description of the following: (i) the nature of the tissue from which the cells were isolated (this also implies to describe whether the cells are respectively autologous, allogeneic or (in experimental studies) xenogeneic), (ii) the isolation procedure itself, including the specific technology that was used, (iii) every process including but not restricted to selecting, cultivating, stimulating, manipulating, (genetically) reprogramming, etc. to which the cells were exposed during the period between isolation and administration into a patient or a model organism, (iv) the exact route of administration, including the total volume of the administered final cell suspension, and (v) every additional therapy that was applied (this also comprises any administration of drugs or other biologics such as platelet rich plasma (PRP) before, during or after administration of cells; c.f., e.g., [5]).

We have strictly followed this route in our recent reports about the application of uncultured, autologous, fresh, unmodified, adipose derived regenerative cells (UA-ADRCs), isolated at point of care (i.e., at the same location where harvesting of adipose tissue and injection of UA-ADRCs were carried out) with the Transpose RT /Matrase system (InGeneron, Houston, TX) [6-8]. In this regard we define treatments with UA-ADRCs as follows:

Firstly, UA-ADRCs are isolated at the point of care from the patient's own adipose tissue, usually harvested by a mini-liposuction (in specific cases adipose tissue can also be harvested by surgical extraction). This clearly differentiates UA-ADRCs from cells that are isolated from respectively bone marrow, umbilical cord tissue, umbilical cord blood or specific organs (such as the isolation of stem cells from tendons, other connective tissue or amniotic or synovial fluid [9]).

Secondly, UA-ADRCs are isolated from adipose tissue such that they are separated from both adipocytes and the connective tissues. In general, one has to differentiate between methods for generating so-called nanofat (described in the literature as mechanically emulsified fat tissue in a liquid

form, ideally devoid of connective tissues but containing cells of the stromal vascular fraction [10]) and methods for isolating only the stromal vascular fraction (i.e., a cellular extract made from fat that is devoid of both adipocytes and connective tissues [11]). The latter can be achieved with or without the use of enzymes, with a much higher cell yield (number of nucleated cells per unit weight of adipose tissue or volume unit lipoaspirate) achieved with enzymatic methods that with non-enzymatic ones [11]. Cells that are isolated from adipose tissue in a way that they are devoid of adipocytes but not of connective tissues (e.g., [12]) should not be called stromal vascular fraction and/or ADRCs.

Thirdly, UA-ADRCs are not cultivated, selected, stimulated, manipulated, (genetically) reprogrammed etc., but administered into the patient's tissue in need for regeneration (e.g., bone defects [6], heart tissue with impaired function as a consequence of previous myocardial infarction [7] or partial tendon ruptures [8], respectively) immediately after isolation of the cells (usually within less than two hours after harvesting of the adipose tissue). Cultivating UA-ADRCs in the laboratory can be applied for isolating adipose derived stem cells (ASCs), which comes along with all the potential, culture-related mechanic and oxidative stress that could affect their safety as a medicinal product [13].

Fourthly, we administer UA-ADRCs locally according to the individual patient's need. In case of bone defects UA-ADRCs can be surgically administered together with a scaffold [6]. For treating heart failure, we recently published a novel procedure for retrograde administration of UA-ADRCs through the heart’s venous system, precisely to the area in need of regeneration, combined with a temporary blockage of the coronary vein at the level of a previous arterial occlusion [7]. In the case of partial tendon ruptures the cells can be directly injected into the damaged site of the tendon [8]. It is obvious that the latter applications require a final cell suspension of small volume, which is achieved with the technology we are using (usually 3 mL).

Fifthly, we do not apply any other treatment together with UA-ADRCs, except for adequate rehabilitation (such as optional outpatient rehabilitation with physical therapy modalities in case of tendon regeneration [8]).

In the following text we present and discuss nine statements about UA-ADRCs (as defined above) and their application in regenerative medicine, reflecting the current state of knowledge in the literature. They are summarized in Table 1.

Table 1. Nine statements about UA-ADRCs and their application in regenerative medicine, reflecting the current state of knowledge in the literature.

What are the rationale and advantages of using UA-ADRCs in regenerative medicine?
1. Serious and adequate clinical research demonstrates that tissue regeneration with UA-ADRCs is safe.
2. Serious and adequate clinical research demonstrates that tissue regeneration with UA-ADRCs is effective.
Why and how shall regenerative cells be isolated from adipose tissue rather than from other tissues, and how shall these cells be characterized?
3. ADRCs are neither 'fat stem cells' nor could they exclusively be isolated from adipose tissue, as ADRCs contain the same adult pluripotent or multipotent (depending on the definition) stem cells that are ubiquitously present in the walls of small blood vessels.
4. The specific isolation procedure used has significant impact on the number and viability of the cells and hence on safety and efficacy of UA-ADRCs.
5. There is no need to further separate adipose-derived stem cells (ASCs) from ADRCs if the latter were adequately isolated from adipose tissue.
6. The minimal definitions of stromal cells as ADRCs established by the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT) are inadequate and misleading, and therefore should be amended.
How do UA-ADRCs exert their function in tissue regeneration?
7. UA-ADRCs have the physiological capacity to adequately regenerate tissue without need for manipulating, stimulating and/or (genetically) reprogramming the cells for this purpose.
8. Tissue regeneration with UA-ADRCs fulfills the criteria of homologous use.
9. A certain challenge in research with UA-ADRCs lays in the fact that labeling the cells would render them modified, and unmodified cells can only indirectly be identified after transplantation in a target tissue.

2. What are the rationale and advantages of using UA-ADRCs in regenerative medicine?

2.1. Statement #1: Serious and adequate clinical research demonstrates that tissue regeneration with UA-ADRCs is safe.

In a position statement recently published by representatives of the U.S. Food and Drug Administration (FDA) in *The New England Journal of Medicine* [14] safety of stem cell treatments was a primary focus. Marks et al. [14] specifically stated that adverse events are probably more common than is appreciated, because there is no reporting requirement when these therapies are administered outside clinical investigations [14]. In fact, a number of serious adverse events related to stem cell treatments were recently published in *The New England Journal of Medicine* [15-17]. These adverse events included development of a glioproliferative lesion of the spinal cord leading to progressive lower back pain, paraplegia and urinary incontinence after intrathecal infusions of putative mesenchymal, embryonic and fetal neural stem cells for the treatment of residual deficits from an ischemic stroke [15], vision loss after intravitreal injection of autologous ADRCs for the treatment of age-related macular degeneration [16], and lethal human herpesvirus 6–related meningoencephalitis, -myocarditis and -interstitial nephritis after allogeneic transplantation of stem cells for chronic lymphocytic leukemia [17]. These and other reports about serious adverse events related to stem cell treatments highlight the need to conduct controlled clinical studies in order to determine whether these cellular therapies are safe and effective for their intended uses. Marks et al. [14] concluded that without such studies, one would not be able to ascertain whether the clinical benefits of such therapies outweigh any potential harms. These authors also stated that although autologous stem

cells may typically raise fewer safety concerns than allogeneic stem cells, their use may be associated with significant adverse events [14] (as demonstrated in [16]).

The outcome of a recent systematic review of reported adverse events in clinical trials on adipose derived cell therapy [18] exemplifies the need to clearly differentiate between the different types of cells derived from adipose tissue, with the aim to apply them in regenerative medicine. The authors of this systematic review identified 70 studies on adipose derived cell therapy involving more than 1400 patients. Twenty out of the 70 studies were used to evaluate thromboembolic safety and mortality, immunological safety and oncological safety. From the nine studies based on which thromboembolic safety and mortality were evaluated, only four were performed with ADRCs, and all of these studies addressed treatment of myocardial infarction (the administration route was transendocardial (two studies), intramyocardial or intracoronary (one study each), respectively). Furthermore, all of the eleven studies based on which immunological safety was evaluated were performed with allogeneic ASCs. In contrast, all of the five studies based on which oncological safety was evaluated were performed with ADCRs and did not address musculoskeletal conditions or heart failure. The administration routes in these studies were subcutaneous (two studies), transurethral, periurethral or into the corpus cavernosum of the penis (one study each), respectively. In case of treatments with ADRCs in the analyses of thromboembolic safety and mortality as well as oncological safety no distinction was made between enzymatically and non-enzymatically isolated cells. The authors concluded that adipose-derived cell therapy has so far shown a favorable safety profile, but safety assessment description has, in general, been of poor quality [18]. Furthermore, they encouraged future studies to maintain a strong focus on the safety profile of cell therapy, so its safeness can be confirmed [18].

For the aforementioned examples of the application of UA-ADRCs (treatment of bone defects [6], of heart failure with retrograde administration of the cells through the heart's venous system to the area in need of regeneration [7] and of partial tendon ruptures [8]) this safety analysis [18] is almost irrelevant. Rather, the safety of any specific combination of the type of administered cells (enyzmatically or non-enzymatically isolated ADRCs, autologous or allogeneic ASCs, etc.), the target tissue and the exact administration route must be separately evaluated.

In this regard we recently performed a prospective, randomized, controlled, first-in-human pilot study on the safety and efficacy of treating symptomatic, partial-thickness rotator cuff tear (sPTRCT) with UA-ADRCs [8]. Specifically, we treated n=11 subjects with symptomatic partial rupture of the supraspinatus tendon with a single injection of UA-ADRCs, and another n=5 subjects suffering from the same condition with a single subacromial corticosteroid injection (all patients had not responded to an initial phase of at least six weeks with physical therapy treatments). All injections were made by a qualified physician under ultrasound guidance. Because of its first-in-human character the entire study was carried out according to strict guidelines set forth by U.S. FDA [14, 19]. Over a period of one year after treatment, for all subjects any illnesses that made it necessary for them to see a physician had to be documented - completely irrespective of whether the illness that occurred was related to the initial treatment or not (e.g., the breaking of a tooth in one subject 164 days post treatment). This complex procedure prevented that only adverse events that are looked for will be found [18] and resulted, for the first time, in a complete risk profile for the treatment of a musculoskeletal disease with UA-ADRCs [8]. Of note, the risks associated with treatment of sPTRCT with UA-ADRCs were no higher than those with corticosteroid treatment; there were no serious complications. However, one subject treated with corticosteroid injection developed a full rotator cuff tear during the course of this pilot study [8]. This pilot study suggested that the use of UA-ADRCs in subjects with sPTRCT is safe. To verify the results of this initial safety pilot study in a larger patient population, a randomized controlled trial on 246 patients suffering from sPTRCT is currently ongoing [20].

2.2. Statement #2: Serious and adequate clinical research demonstrates that tissue regeneration with UA-ADRCs is effective.

In the aforementioned position statement recently published by representatives of U.S. FDA in *The New England Journal of Medicine* [14] it was stated that the literature is replete with instances of therapeutic interventions pursued on the basis of expert opinion and patient acceptance that ultimately proved ineffective or harmful when studied in well-controlled trials comparing them with the standard of care [14]. In this regard another recent systematic review focused on the efficacy of treatments using ADRCs [21]. The authors identified 73 related clinical studies, of which 12 (16.5%) were randomized controlled trials (RCTs) (defined as Evidence Based Medicine (EBM) Level II in [21]), 14 (19.2%) were cohort studies (EBM Level III in [21]) and 47 (64.4%) were case series (EBM Level IV in [21]). Case series and cohort studies are important to determine whether a novel treatment is effective and should be considered for further investigation. However, the only way to reduce certain sources of bias is testing the effectiveness of new treatments in RCTs against no treatment, a conventional treatment or a placebo. We therefore restrict our analysis to the RCTs identified in [21].

Two out of the 12 RCTs listed in [21] should not be considered RCTs in a strict sense. In one of these studies [22] n=16 subjects with bilateral knee osteoarthritis were treated with UA-ADRCs on one side and with hyaluronic acid (HA) injection on the other side; allocation of either side to UA-ADRCs or HA was performed randomly. In another study [23] subjects suffering from Achilles tendinopathy were randomly allocated to respectively treatment with UA-ADRCs (n=21) or treatment with PRP (n=23). However, for evaluating treatment success using diagnostic ultrasound and magnetic resonance imaging (MRI) all subjects were pooled into one group (n=44) and no comparisons between the different treatments were performed.

Four other RCTs were excluded from further consideration. In one of them ([24]; focussing on treatment of recalcitrant chronic leg ulcers) centrifuged adipose tissue rather than ADRCs was applied. The other three RCTs that were excluded addressed myocardial infarction [25-27]. They were excluded because in the APOLLO trial [25] the mean left ventricular ejection fraction (LVEF) was 52% at baseline which is considered an incorrect target population [28]; in the PRECISE trial [26] the LVEF was not investigated with cardiac MRI which is considered the state-of-the-art for accurate, comprehensive and reproducible measurements of cardiac chamber dimensions, volumes, function and infarct size [28]; and the ATHENA trial [27] was initially put on hold because of delivery related cerebrovascular events [7] and afterwards terminated prematurely due to subsequent prolonged enrollment time [27].

The remaining six RCTs are summarized in Table 2. In only one of them (addressing Achilles tendinopathy [29]) UA-ADRCs were applied as the sole therapy. The same study was the only one in which a commercially available (non-enzymatic) method was used for isolating ADRCs (FastKit; Corios, San Giuliano Milanese, Italy). Only short-term benefits of injecting UA-ADRCs compared to injection of PRP were observed in this study (statistically significantly lower mean VAS pain scores at 15 and 30 days (D15 and D30) post treatment; statistically significantly higher mean VISA-A score on D30 post treatment; and statistically significantly higher mean AOFAS score on D15 post treatment), but no long-term benefits (i.e., no statistically significantly differences between the groups on D60, D120 and D180 post treatment) [29]. In the other five RCTs listed in Table 2 ADRCs were isolated with experimental, commercially not available (enzymatic) methods, and were not used as the sole therapy. Furthermore, in none of these six RCTs a safety profile comparable to the one established in our recent pilot study on treating sPTRCT with UA-ADRCs [8] was established.

Table 2. RCTs for treating various conditions with UA-ADCRs reported in the literature (c.f. [21]).

Study	[29]	[30]	[31]	[32]	[33]	[34]
Indication	Achilles tendinopathy	Knee OA	Knee OA	Skin ulcers	Skin ulcers	Painful amputation stump
N (Tr)	21	26	30	28	16	5
Tr	UA-ADRCs	HTO + UA-ADRCs + PRP	MF + UA-ADRCs	UA-ADRCs + Fibrin	UA-ADRCs + PRP	UA-ADRCs + Fat
I-PoC	Yes	No (1 day before)	No (1 day before)	Yes	Yes	Yes
N (C)	23	26	27	26	24	5
C	PRP	HTO + PRP	MF	Fibrin	PRP	Fat
E / NE	NE	E	E	E	E	E
Follow-up	M6	M24	M26-M30	W8	M18	M6
Tr > C	No	Yes	Yes*	Yes	No**	No

Abbreviations: OA, osteoarthritis; N (Tr), number of subjects in the treatment group; Tr, treatment; HTO, high tibial osteotomy, UA-ADRCs, uncultured, autologous, adipose derived regenerative cells; HTO, open-wedge high tibial osteotomy; PRP, platelet rich plasma; MF, microfractures; I-PoC, isolation of ADRCs at the point of care; N (C), number of subjects in the control group; C, control treatment; E / NE, enzymatic / non-enzymatic isolation of ADRCs; M, month; W, week; Tr > C, outcome after treatment superior to outcome after control treatment. *, only short-term effects (c.f. main text); ** when all subjects were considered that were enrolled in this study.

This overall very limited evidence base stands in contrast to the high number of enzymatic and non-enzymatic methods for isolating ADRCs that are commercially available (addressed in detail in Section 3.2). Furthermore, in case the so-called 'stem cell' preparations that are recommended, prescribed or delivered in many clinical centers around the world (most probably more than 1.000 in the U.S. [35]) are ADRCs, these treatments are indeed performed without sufficient data to support their true efficacy [36], supporting U.S. FDA's warnings about these stem cell therapies [37].

We consider our recent prospective, randomized, controlled, first-in-human pilot study on the safety and efficacy of treating sPTRCT with UA-ADRCs ([8]; described in detail in Section 2.1) as a first, important step to overcome this unsatisfactory situation. Despite the small number of subjects in this pilot study, those in the UA-ADRCs group (n=11) showed statistically significantly higher mean American Shoulder and Elbow Surgeons Standardized Shoulder Assessment Form total scores (ASES total scores) both 24 and 52 weeks post treatment than those in the corticosteroid group (n=5). The ASES total score takes into account the patient's pain situation and the functionality of the shoulder [38, 39]. As already mention above a randomized controlled trial on 246 patients suffering from sPTRCT is currently ongoing to verify the results of this pilot study [20].

3. Why and how shall regenerative cells be isolated from adipose tissue rather than from other tissues, and how shall these cells be characterized?

3.1. Statement #3: ADRCs are neither 'fat stem cells' nor could they exclusively be isolated from adipose tissue, as ADRCs contain the same adult (depending on the definition) pluripotent or multipotent stem cells that are ubiquitously present in the walls of small blood vessels.

One of the greatest misconceptions in stem cell-based regenerative medicine may be the designation of ADRCs as 'fat stem cells' and related descriptions in the recent literature (e.g. 'adipose-derived stem cells: fatty potentials for therapy' [40], 'using fat to fight disease' [41], 'adipose tissue stem cells for therapy' [42] and 'stem cells derived from fat' [43]). In fact, these cells are not 'fat stem cells' at all. Rather, they are adult (depending on the definition) pluripotent or multipotent stem cells located in the walls of small blood vessels (henceforth vascular associated mesenchymal stem cells (MSCs)) (reviewed in [1, 4]). Because blood vessels are stimulated to grow, branch and invade developing tissues and organs very early during human embryonic development (starting on approximately Day 18 [44]) the presence of vascular associated MSCs in the vascular location results in equal distribution of these cells throughout the body. As a result, vascular associated MSCs can in principle also be isolated from small blood vessels in other organs (shown for heart and skeletal muscle in [4]).

The reason why vascular associated MSCs are isolated from adipose tissue is that the latter contains a large amount of small blood vessels and is relatively easy to be harvested in most patients through liposuction. Furthermore, vascular associated MSCs can represent up to 12% of the total population of SVF cells [7], whereas only 0.001–0.1% of the total population of bone marrow nucleated cells represent MSCs [45, 46]. Besides this, harvesting adipose tissue (by liposuction) is typically much less invasive than harvesting bone marrow [40, 45, 47]. Approximately 400.000 elective liposuction surgeries are performed in the U.S. per year [48], with a serious adverse event rate reported between 0.07% and 0.7% [49, 50].

Another misconception is the belief that microvascular pericytes are the vascular associated MSCs in the walls of small blood vessels (e.g., [1, 51, 52]). This misconception is based on the fact that expression of the proteoglycan neural/glial antigen 2 (NG2) has long been associated with pericytes [51-53]. In the central nervous system (CNS), NG2-positive cells are responsible for the generation of oligodendrocytes [54]. Some authors presented results suggesting that even astrocytes and neurons may be generated from NG2-positive cells, which would make the latter similar to neural stem cells [54]. However, other authors could not reproduce these findings [55]. It is of note that different populations of pericytes and pericyte-like progenitor cells were described in the literature. Most probably the best studied pericytes are parts of typical capillary structures, formed by pericytes and endothelial cells [56-58]. Another type of pericytes is located at the surface of small blood vessels, partly taking over regulation of vessel diameter and, thus, hemodynamic regulation in the CNS [59]. Pericyte-like progenitor cells were also described in the adventitia of larger vessels [60]. However, all of these types of pericytes have positions in the wall of capillaries or larger vessels that are clearly distinct to the position of NG2-positive cells in the wall of a small human arteriole shown in Figure 1a. This indicates that NG2 is expressed by more cells than just pericytes, and it is much more likely that vascular associated MSCs are also immunopositive for NG2 [4]. Figure 1b shows the current concept of the localization of vascular associated MSCs in the wall of small vessels.

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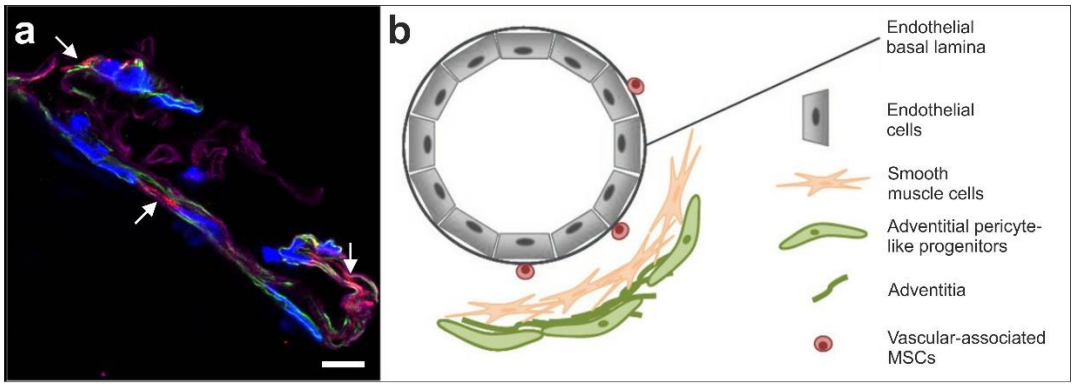

Figure 1. (**a**) Immunofluorescent detection of NG2 (red; arrows), smooth muscle antigen (SMA) (green) and laminin (purple) in the wall of a small human arteriole; cell nuclei are in blue (modified from [4]). The scale bar represents 10 µm. (**b**) Schematic illustration of the hypothesized location of vascular associated MSCs (taken from [4] and modified from [61]).

3.2. Statement #4: The specific isolation procedure used has significant impact on the number and viability of the cells and hence on safety and efficacy of UA-ADRCs.

It is important for physicians and patients to understand that terms such as 'UA-ADRCs', 'fat stem cells', 'stromal vascular fraction', 'SVF' etc. are only generic terms for cell preparations which are isolated from the patient's own adipose tissue immediately before transplantation into the target tissue. In fact, an optimal technology for isolating ADRCs should be able to isolate the highest possible number of living ADRCs in the shortest possible time from the smallest possible amount of adipose tissue/lipoaspirate, and should result in the highest possible concentration of cells in the final cell suspension (i.e. the smallest possible volume of cell suspension) for immediate application. Various enzymatic and non-enzymatic methods were developed for this purpose, some of which are available on the market (reviewed in [11, 62-65]). Using enzymatic methods the connective tissue of the adipose tissue and the walls of the small blood vessels are largely dissolved, resulting in a cell yield (i.e., number of isolated ADRCs) that is on average many times higher than the cell yield achieved with non-enzymatic methods in which the cells are isolated purely mechanically [11] (Fig. 2a). In addition, for the vast majority of non-enzymatic methods described in literature, no data are available on the relative number of living cells (or the extent of cell death due to the mechanical processing of the adipose tissue) in the final cell suspension [11]. However, these aspects are of immediate clinical relevance, because (i) the transplantation of an insufficient number of UA-ADRCs can lead to an unsatisfactory clinical result, and it does not appear medically justifiable to remove much more adipose tissue from the patient simply because (for whatever reason) a non-enzymatic method for isolating ADRCs is used; and (ii) injection of dying cells into tissue can lead to inflammatory reactions [66]. Considering the limited tendency particularly of tendons to heal, undesirable side effects of any kind should be avoided as far as possible in tissue regeneration.

We have recently demonstrated that isolating ADRCs from adipose tissue using the Transpose RT / Matrase system (InGeneron) results in a high cell yield ($7.2\times10^5 \pm 0.90\times10^5$ ADRCs per mL lipoaspirate in [11]), high cell viability (85.9% ± 1.1% in [11]) and, thus, high number of living cells per mL lipoaspirate ($6.25\times10^5 \pm 0.79\times10^5$ ADRCs per mL lipoaspirate in [11]). To our knowledge the latter is the highest value ever reported in studies describing methods for isolating ADRCs [11] (Fig. 2b). Figure 3 provides a schematic representation of isolating ADRCs from lipoaspirate with the Transpose RT / Matrase system (InGeneron).

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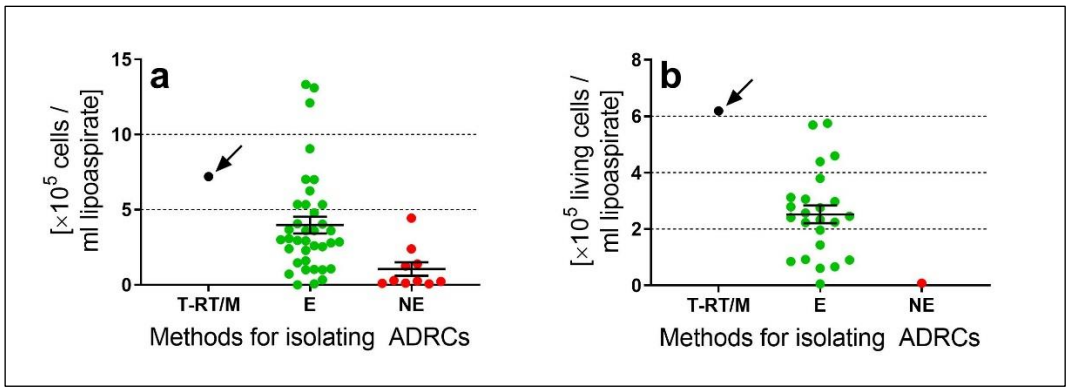

Figure 2. Comparison of cell yield (**a**) and number of living cells per mL lipoaspirate (**b**) of ADRCs isolated with the Transpose RT / Matrase system (InGeneron) (T-RT/M; black dots) as well as other enzymatic (E; green dots) and non-enzymatic (NE; red dots) methods for isolating ADRCs reported in the literature (modified from [11]). The panels show individual data (dots) as well as mean and standard error of the mean (SEM) in case of the other enzymatic and non-enzymatic methods. One can immediately see that on average enzymatic methods result in a much higher cell yield than non-enzymatic methods. Furthermore, for most of the non-enzymatic methods the number of living cells per mL lipoaspirate could not be calculated because the corresponding relative numbers of living cells were not reported. Of all reported methods the Transpose RT / Matrase system (InGeneron) did not result in the highest cell yield (arrow in a) but in the highest number of living cells per mL lipoaspirate (arrow in b), which appears to be the clinically most relevant parameter (as outlined in the main text).

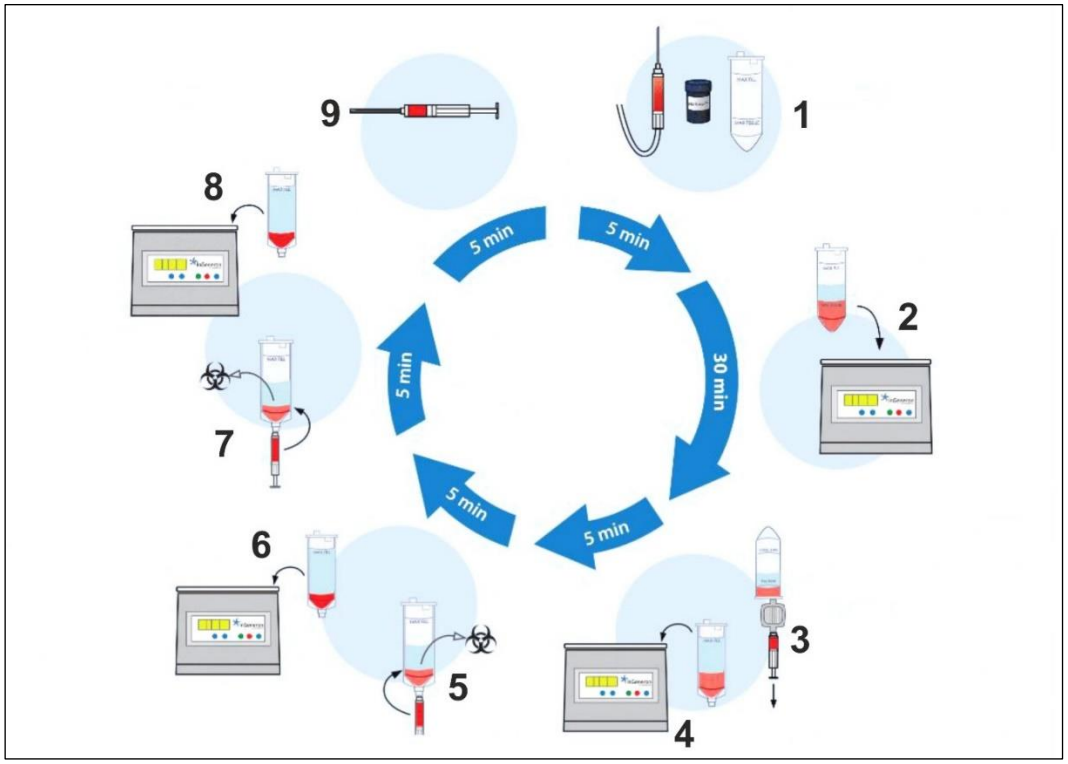

Figure 3. Schematic representation of isolating UA-ADRCs from lipoaspirate with the Transpose RT / Matrase system (InGeneron) (derived from [11]): (1) recovered lipoaspirate (25 mL) is loaded together with 2.5 mL reconstituted, proprietary enzymatic Matrase Reagent and lactated Ringer solution (preheated to 39° C) into a processing tube up to the MAX FILL line; (2) the filled processing tubes are subjected in an inverted position inside the Transpose RT system to repetitive acceleration and deceleration for 30 minutes at 39° C; (3) the processed lipoaspirate solution is filtered through a 200 µm filter and transferred into a wash tube; (4) after filling the wash tube with saline (room temperature) up to the MAX FILL line, the cells are separated from the rest of the tissue by

centrifugation at 600g for 5 minutes at room temperature; (5) the ADRCs (approximately 2 mL) are extracted through a swabable luer vial adapter at the bottom of the wash tube, and the remaining substances (fat, debris and liquid) are discarded; (6) the cells are returned into the empty wash tube and (after adding fresh saline up to the MAX FILL line) centrifugated again for 5 minutes; (7, 8) the previous washing step is repeated; and (9) finally the concentrated ADRCs (approximately 3 mL) are extracted and slowly pushed through a luer coupler into a new sterile syringe for further application to the subject.

3.3. Statement #5: There is no need to further separate adipose-derived stem cells (ASCs) from ADRCs if the latter were adequately isolated from adipose tissue.

Figure 4 provides a schematic overview of the relationship between the terms adult stem cells, vascular associated MSCs, ADRCs and ASCs. Vascular associated MSCs are a subgroup of adult stem cells and are contained in ADRCs. ASCs can be obtained by culturing ADRCs and, thus, selectively propagating the vascular associated MSCs contained in ADRCs. For example, it was shown that culturing ADRCs increased the mean relative number of cells immunopositive for the surface marker CD29 (a marker of ASCs [65, 67]) (CD29+ cells) from 71% at passage 1 to 95% at passage 4, and the mean relative number of CD44+ cells (another marker of ASCs [65, 67]) from 84% at passage 1 to 98% at passage 4 [68]).

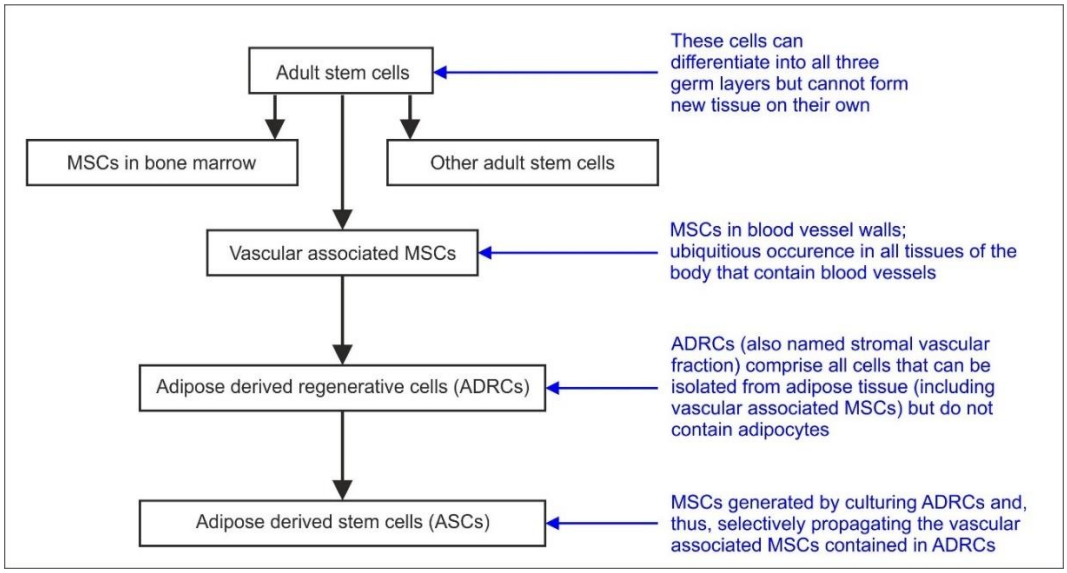

Figure 4. Schematic overview of the relationship between the terms adult stem cells, vascular associated MSCs, ADRCs and ASCs (modified from [4]).

Considering the fact that ADRCs also contain other types of cells next to vascular associated MSCs (among them blood-derived cells, endothelial cells and pericytes [7]) one could be inclined to believe that ASCs may be the better choice for tissue regeneration than ADRCs. For the following reasons, however, this is not the case:

Firstly, cultivating ADRCs inevitably results in exposure to potential, culture-related mechanic and oxidative stress that could affect the safety of ASCs as a medicinal product [13]. As a result, ASCs may not meet the criterion 'minimally manipulated' defined in 21 CFR 1271.10(a) [69] (Title 21 is the portion of the Code of Federal Regulations that governs food and drugs within the U.S. for the FDA [70]) and, thus, may not be regulated solely under Section 361 of the Public Health Service (PHS) Act and 21 CFR Part 1271 [71]. Rather, ASCs may be regulated as a drug, device and/or biologic product under the Federal Food, Drug and Cosmetic (FD&C) Act and/or Section 351 of the PHS Act (and applicable regulations) in the U.S. [71]. The European Medicines Agency may consider ASCs as an Advanced Therapy Medicinal Product (ATMP) [72].

Secondly, a number of recent studies on culture systems and animal models has indicated non-inferiority or even superiority of UA-ADRCs over ASCs in rescuing heart function after acute

myocardial infarction [73] as well as in tendon regeneration [74], bone regeneration [75] and erectile function recovery after cavernous nerve injury [76] (see also [77]). It is currently unknown whether this is due to alterations of the physiological functions of vascular associated MSCs when they are (as ASCs) exposed to culture-related mechanic and oxidative stress, or due to the fact that ADRCs comprise more cells than just ASCs which may act synergistically in regenerating tissue.

3.4. Statement #6: The minimal definitions of stromal cells as ADRCs established by the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT) are inadequate and misleading, and therefore should be amended.

In a position statement published by ISCT in 2006 the following minimal criteria for defining multipotent MSCs were described: being adherent to plastic, expressing the surface markers CD73, CD90 and CD105, and having the ability to differentiate into osteoblasts, adipocytes and chondrocytes [78].

This definition has a number of shortcomings. Most importantly, fibroblasts are also adherent to plastic and express CD73, CD90 and CD105, but are not MSCs and cannot transdifferentiate into other lineages [79]. Furthermore, the true pluripotent stem cells do not yet express CD73, CD90 and CD105 [4]. Rather, expression of cell surface markers is a dynamic process. For example, when cultured in fetal bovine serum or platelet lysate culture media, MSCs can turn on new surface markers [4]. Alternatively, they can turn down surface markers in culture, such as the loss of the previously expressed progenitor marker CD34 or the endothelial progenitor marker CD31 [4].

In another position statement published by IFATS and ISCT in 2013 regarding SVF and culture-expanded ASCs it was stated that primary stable positive surface markers for stromal cells are CD13, CD29, CD34 (>20%), CD44, CD73 and CD90 (>40%), whereas primary negative surface markers for these cells are CD31 (<20%) and CD45 (<50%) [80]. Furthermore, at least 20% of the SVF cells would be CD31-/CD34+/CD45-/CD235a- [80].

We recently performed a comprehensive analysis of relative amounts of ADRCs expressing these surface markers (i.e. CD13, CD29, CD34, CD44, CD73, CD90, CD31 and CD45) as reported in studies describing enzymatic and non-enzymatic methods for isolating ADRCs [11] and found the following. Firstly, for only very few methods [68, 81-83] the relative amount of CD34+ ADRCs was determined, with substantial variation among methods (range, 35% - 81%). Secondly, for most methods CD34 was determined together with at least one other surface marker, resulting in a large range of published data between 0.8% (CD34+/CD90-/CD31-/CD45-/CD105-/CD146+ cells; [84]) and 44% (CD34+/CD31-/CD45- cells; [85]). Thirdly, the relative amount of CD45+ ADRCs varied between 6% [86] and 50% [82] for enzymatic methods, and between 8% [86] and 82% [83] for non-enzymatic methods. Fourthly, for only a few methods the relative amounts of CD13+ cells, CD29+ cells, CD44+ cells, CD73+ cells, CD90+ cells and CD31+ cells were determined [11]. Fifthly, the relative amount of CD31-/CD34+/CD45-/CD235a- cells (as proposed in [80]) was not determined for any published method. Sixthly, no correlation was found between the cell yield obtained with a certain method and any single surface marker or any combination of surface markers, respectively [11].

Taken together, the vast majority of reported methods for isolating ADRCs were not characterized according to the position statements published by IFATS and ISCT [78, 80]. Considering the large range of those few data that were reported in this regard it appears reasonable to hypothesize that determining surface markers of ADRCs is in principle not suitable for characterizing a method for isolating ADRCs. In our opinion any method for isolating ADRCs should primarily be assessed by the safety and efficacy of clinical applications of the isolated cells, determined by adequate clinical trials (pilot studies followed by RCTs).

4. How do UA-ADRCs exert their functions in tissue regeneration?

4.1. Statement #7: UA-ADRCs have the physiological capacity to adequately regenerate tissue without need for manipulating, stimulating and/or (genetically) reprogramming the cells for this purpose.

In the aforementioned position statement recently published by representatives of U.S. FDA in *The New England Journal of Medicine* [14] it was stated that outside the setting of hematopoietic reconstitution and a few other well-established indications, the assertion that stem cells are intrinsically able to sense the environment into which they are introduced and address whatever functions require replacement or repair – whether injured knee cartilage or a neurologic deficit – is not based on scientific evidence [14]. The following two examples from our own published clinical research using UA-ADRCs may give a different picture.

The first example (described in detail in [6] is a male, 79-year old patient who presented with a partly failing maxillary dentition (yellow arrows in Fig. 5a) and was treated with a bilateral external sinus lift procedure as well as a bilateral lateral alveolar ridge augmentation (called 'guided bone regeneration / maxillary sinus augmentation / lateral alveolar ridge augmentation'; henceforth: GBR-MSA/LRA). On the right side GBR-MSA/LRA was performed with a combination of UA-ADRCs, fraction 2 of plasma rich in growth factors (PRGF-2) and an osteoinductive scaffold (OIS) (Treatment A). On the left side GBR-MSA/LRA was performed with the same combination of PRGF-2 and OIS but without UA-ADRCs (Treatment B). Accordingly, the only difference between the treatments was the presence (Treatment A) or absence (Treatment B) of UA-ADRCs. Biopsies were collected at six weeks and 34 weeks post treatment. At the latter time point implants were placed. Radiographs (6 weeks and 32 months post treatment) demonstrated excellent bone healing (yellow arrows in Fig. 5b; Fig. 5c). No radiological or histological signs of inflammation were observed. Detailed histologic, histomorphometric and immunohistochemical analysis of the biopsies evidenced that Treatment A resulted in better and faster bone regeneration than Treatment B. Specifically, Treatment A resulted in faster build up of higher relative amounts (area/area) of newly formed bone, connective tissue and arteries as well as in lower relative amounts of adipocytes and veins at 34 weeks after GBR-MSA/LRA than Treatment B (Fig. 5d-f).

The second example (described in detail in [4]) is a male, 51-year-old patient who presented with recurring and increasing pain in both knee joints during walking and other activities. The patient's history included a tibial chondrocyte transplant that had been performed three years previously. Figure 6a shows an arthroscopic view of third-degree damage to the right tibial plateau where the transplanted chondrocytes were gone and only the artificial matrix with small holes implanted on the tibial plateau was still present (white asterisk in Fig. 6a). Furthermore, considerable osteoarthritic damage of the femoral cartilage was observed (black asterisk in Fig. 6a). Figure 6b shows the situation after arthroscopic removal of the failed chondrocyte transplant (white asterisk in Fig. 6b) as well as 'mushy' and damaged cartilage structure on the femoral condyles before it was removed (black asterisk in Fig. 6b). Then, the right knee was treated with a single application of UA-ADRCs, whereas the left knee was treated with a standard therapy, i.e., arthroscopic removal of damaged cartilage and drilling of small holes into the bone. Control arthroscopies were performed one year later. On the right side (treated with UA-ADRCs) complete healing of the tibial defect (white asterisk in Fig. 6c) and of the femoral parts (black asterisk in Fig. 6c) was observed, with formation of new whitish cartilage that showed a sharp demarcation border to the original, more yellowish cartilage (arrows in Fig. 6c). In contrast, a somewhat uneven, overshooting fibroblastic scar formation was found on the left side (treated with a standard therapy) (asterisk in Fig. 6d), without a sharp demarcation border to the original cartilage (arrows in Fig. 6d). This indicated that there was some sort of healing, but not a regrowth of organized cartilage, as we hypothesized for the right knee after application of UA-ADRCs. Small biopsies that were taken from the regenerated tissue during the follow-up arthroscopies showed the following. After application of UA-ADRCs there was newly formed cartilage with a zonal organization and (like in a textbook of histology) differently shaped chondrocytes in a superficial layer (SL in Fig. 6e), middle layer (ML in Fig. 6e) and deep layer (DL in Fig. 6e). Furthermore, the contact zone between the newly formed cartilage and bone showed (also

like in a textbook of histology) typical chondrocytes with a small nucleus and a hollow space around (arrows in Fig. 6f). In contrast, after treatment with a standard therapy there was a more amorphous fibrocartilage with scattered cells (arrows in Fig. 6g) but without layered organization, and the contact zone between the newly formed cartilage and bone showed an infiltration with inflammatory cells, fibroblasts (arrows in Fig. 6h) and small blood vessels (arrowheads in Fig. 6h).

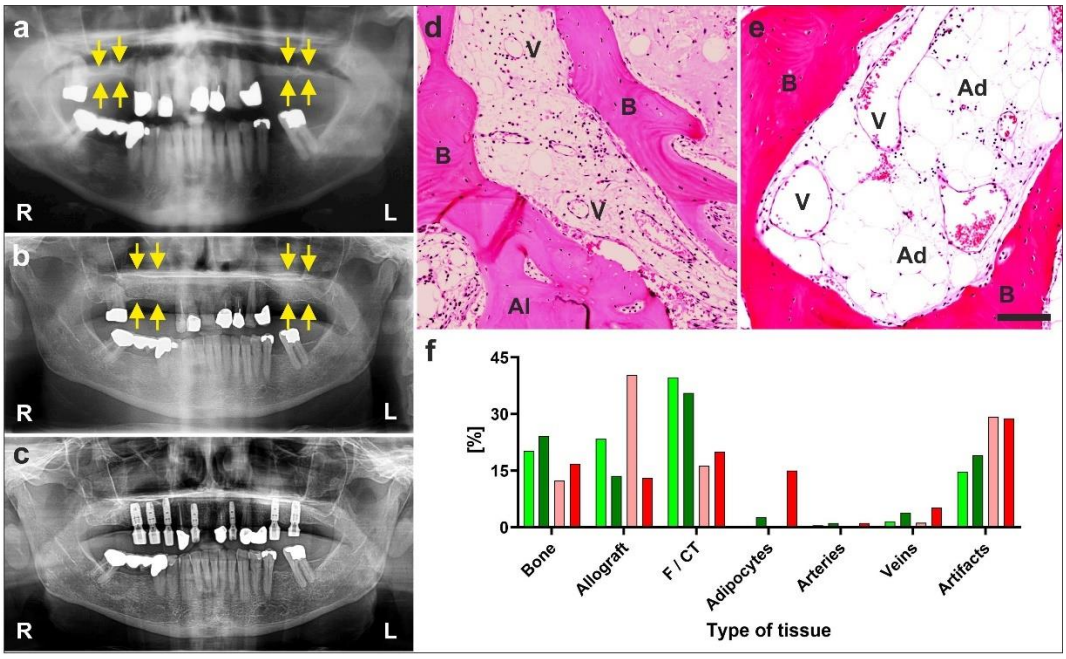

Figure 5. Example of regeneration of bone with UA-ADRCs (modified from [6]). Details are provided in the main text. Abbreviations: R, right; L, left; B, bone; Al, allograft; V, vein; Ad, adipocyte; F / CT, fibrin and connective tissue. In (f) the green bars represent data obtained on the right side (Treatment A with UA-ADRCs) six weeks (light green bars) and 34 weeks (dark green bars) post treatment, and red bars data obtained on the left side (Treatment B without UA-ADRCs) six weeks (light red bars) and 34 weeks (dark red bars) post treatment. With cells there was considerably more bone and connective tissue formed already at six weeks than was achieved without cells even after 6 month. The scale bar in f represents 100 µm.

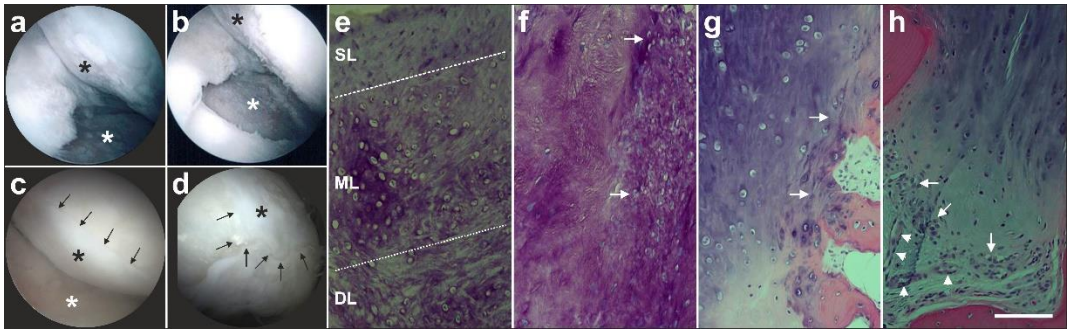

Figure 6. Example of regeneration of knee cartilage with UA-ADRCs (modified from [4]). Details are provided in the main text. The scale bar in h represents 100 µm

Accordingly, in both examples the application of UA-ADRCs resulted in better and more adequate tissue regeneration than a standard therapy. Importantly, this was achieved in both examples without any manipulation, stimulation and/or (genetically) reprogramming of the UA-ADRCs prior to transplantation. As a result, these examples demonstrate that UA-ADRCs are indeed intrinsically able to sense the environment into which they are introduced and adequately regenerate tissue. Related examples from the fields of wound healing and tendon regeneration can be found in [87, 88].

4.2. Statement #8: Tissue regeneration with UA-ADRCs fulfills the criteria of homologous use.

According to 21 CFR 1271.3(c), homologous use means the repair, reconstruction, replacement, or supplementation of a recipient's cells or tissues with a human cell, tissue, and cellular and tissue-based product (HCT/P) that performs the same basic function or functions in the recipient as in the donor [69]. As an example, FDA's regulatory considerations for HCT/Ps define transplantation of a heart valve to replace a dysfunctional heart valve homologous use because the donor heart valve performs the same basic function in the donor as in the recipient (i.e., ensuring unidirectional blood flow within the heart) [71]. On the other hand, the same regulatory considerations by FDA specify that HCT/Ps from adipose tissue used to treat musculoskeletal conditions such as arthritis or tendonitis (by regenerating or promoting the regeneration of articular cartilage or tendon) are generally not considered homologous use because regenerating or promoting the regeneration of cartilage or tendon is not a basic function of adipose tissue [71].

However, regeneration is not based on adult tissue such as adipose tissue, but on the presence of the ubiquitously distributed small universal stem cell. Based on the following evidence we hypothesize that future research will demonstrate that regeneration of musculoskeletal tissue is indeed a basic function of the stromal vascular fraction (and, thus, HCT/Ps from adipose tissue used to treat musculoskeletal conditions should be considered homologous use).

Firstly, ADRCs can induce the formation of new blood vessels in adipose tissue [89] as well as in bone [6], ischemic myocardium [7] and other target tissues [90]. Accordingly, application of ADRCs with the aim to induce the formation of new blood vessels fulfills the criterion of the same basic function or functions in the recipient as in the donor and, thus, should be considered homologous use.

Secondly, it is well known that various pathological conditions result in mobilization of stem cells into the peripheral blood. For example, in the peripheral blood of patients suffering from Crohn's disease [91] or skin burn injury [92] higher mean numbers of cells expressing markers for MSCs, endothelial progenitor cells and very small embryonic-like stem cells (VSELSCs) were found than in the peripheral blood of age-matched controls. Other studies demonstrated mobilization of stem cells into the peripheral blood after acute myocardial infarction in both patients [93] and an animal model [94], and after Achilles tendon transection in an animal model [95].

These data raise the question about the origin of these stem cells. In the aforementioned studies on patients suffering from Crohn's disease or skin burn injury [91, 92] VSELSCs were characterized as immunopositive for a number of stem cell markers (i.e., Oct-4+ [96], Nanog+ [97], SSEA-4+ [98] and CXCR4+ [99]) and immunonegative for hematopoietic markers (i.e., Lin- and CD45- [100]). These VSELSCs also were found in adult bone marrow [101]. Furthermore, Oct-4, Nanog, SSEA-4 and CXCR4 were reported to be also expressed by ASCs [11, 102-105]. Based on our previous research, we consider ADRCs or ASCs to comprise Oct-4+/Nanog+/SSEA-4+/CXCR4+/CD45- cells. However, the minimal definitions of stromal cells as ADRCs established by IFATS and ISCT ([80]; c.f. Section 3.4) do not consider markers such as Oct-4, Nanog, SSEA-4 and CXCR4 to be specific for stemness.

However, a recent study found that one day after induction of acute myocardial infarction (AMI) in rats the number of ASCs was significantly reduced in the stromal vascular fraction compared to healthy control animals, without alterations in the cell surface marker profile and the differentiation capacity of the ASCs [106]. The authors of this study hypothesized that the decreased number of ASCs after AMI could be the result of mobilization of vascular associated MSCs from adipose tissue into the peripheral blood.

Collectively one can hypothesize on the basis of these data that isolating ADRCs from a patient's adipose tissue and transplanting them as UA-ADRCs into the same patient's target tissue in need of regeneration may represent augmentation of a physiological process that also runs to a lesser extent on its own. It will be the task of future research to test this hypothesis. If this hypothesis turns out to be correct, application of ADRCs should be fully considered homologous use, because regenerating or promoting the regeneration of musculoskeletal tissue would indeed be a basic function of a certain component of adipose tissue.

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4.3. Statement #9: A certain challenge in research with UA-ADRCs lays in the fact that labeling the cells would render them modified, and unmodified cells can only indirectly be identified after transplantation in a target tissue.

With regard to the potential mechanisms of action of UA-ADRCs in tissue regeneration it is crucial to bear in mind that, in contrast to ASCs, UA-ADRCs in principle cannot be labeled. Accordingly, it is not possible to experimentally (or even clinically) determine whether the following benefits of ASCs also apply to UA-ADRCs, although it is reasonable to hypothesize that this is indeed the case. Specifically, it has been demonstrated that ASCs can stay locally, survive and engraft in the new host tissue into which the cells were applied [107] (an example is shown in Fig. 7), differentiate under guidance of the new microenvironment into cells of all three germ layers [11], integrate into and communicate within the new host tissue by forming direct cell-cell contacts [4], exchange genetic and epigenetic information through release of exosomes [4], participate in building new vascular structures in the host tissue [4, 6, 7] (c.f. Fig. 7), positively influence the new host tissue by release of cytokines (among them vascular endothelial growth factor and insulin-like growth factor 1) [108], protect cells at risk in the new host tissue from undergoing apoptosis [108-110] and induce immune-modulatory and anti-inflammatory properties [111, 112]. Most probably the combination of these mechanisms of action render UA-ADRCs a powerful tool in tissue regeneration.

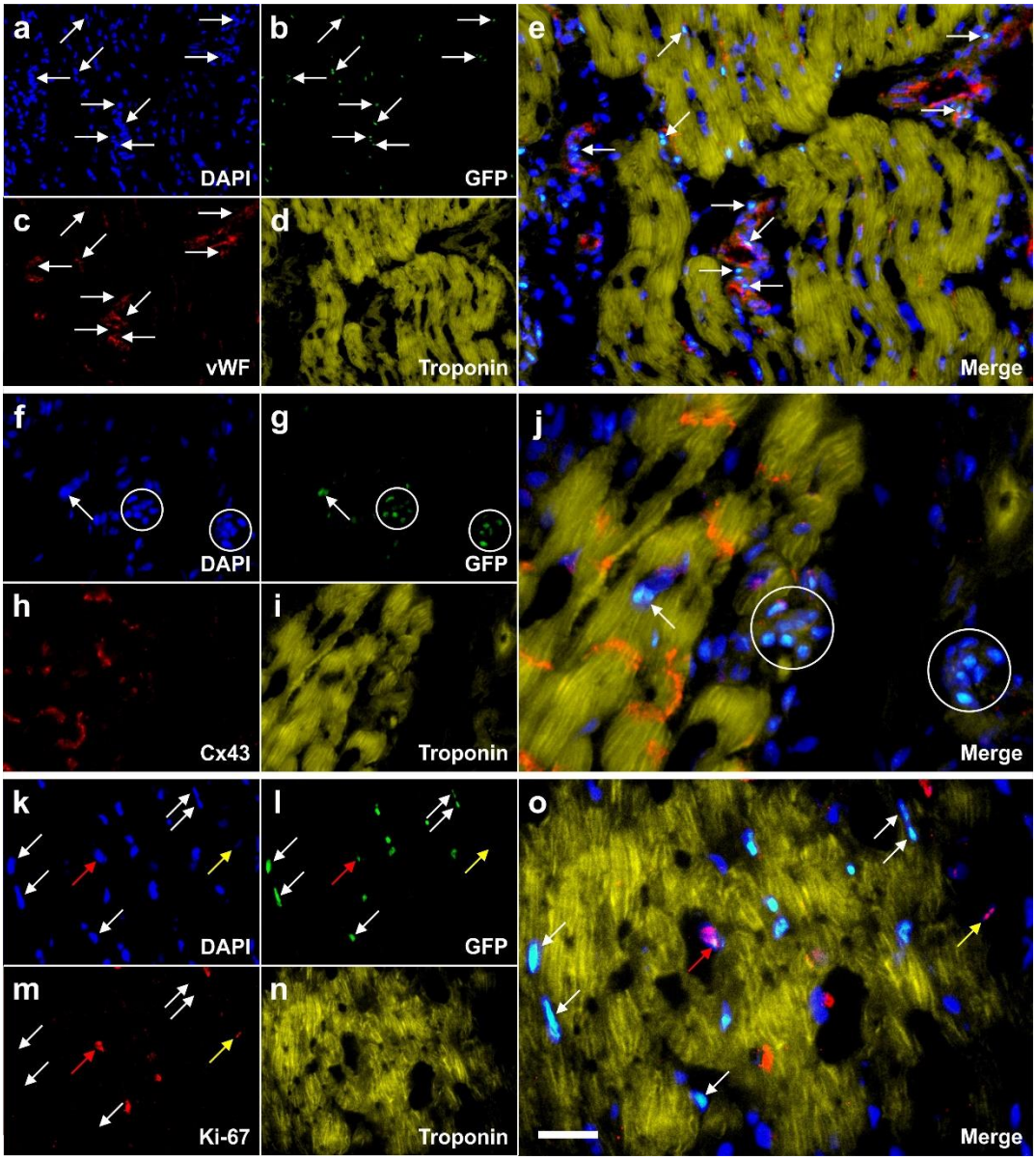

Figure 7. Representative example how autologous, adipose derived stem cells can stay locally,

survive and engraft in the new host tissue into which the cells were applied, differentiate under guidance of the new microenvironment, integrate into the new host tissue and participate in building new vascular structures in the host tissue. The panels show photomicrographs of paraffin-embedded, 5 μm thick tissue sections of a post mortem heart from a pig, taken from the left ventricular border zone of myocardial infarction ten weeks after experimental occlusion of the left anterior descending (LAD) artery for three hours, followed by delivery of eGFP-labeled autologous ASCs into the balloon-blocked LAD vein (matching the initial LAD occlusion site) at four weeks after occlusion of the LAD (experiments are described in detail in [7]). (**a**-**e**) One tissue section was stained with DAPI (blue) (a) and processed for immunofluorescent detection of GFP (green) (b), von Willebrand factor (vWF) (red) (c) and Troponin (yellow) (d). The arrows indicate cell nuclei that were immunopositive for GFP and were found in the wall of small vessels (the positions of these cell nuclei are also labeled in the panel representing vWF). (**f**-**j**) Another tissue section was was stained with DAPI (blue) (f) and processed for immunofluorescent detection of GFP (green) (g), Cx43 (red) (h) and Troponin (yellow) (i). The circles indicate regions where most of the cell nuclei were immunopositive for GFP, and the arrow a GFP-positive cell nucleus inside (or directly adjacent to) a cardiomyocyte. (**k**-**o**). A third tissue section was stained with DAPI (blue) (k) and processed for immunofluorescent detection of GFP (green) (l), Ki-67 (red) (m) and Troponin (yellow) (n). The white arrows point to cell nuclei that were immunopositive for GFP but not for Ki-67, the yellow arrows to a cell nucleus that was immunopositive for Ki-67 but not for GFP, and the red arrows to a cell nucleus that was immunopositive for both GFP and Ki-67 (indicating that this cell had re-entered the cell cycle). The scale bar represents 25 μm in the merged panels and 50 μm in the individual panels.

For labeling cells with eGFP cells isolated from subcutaneous adipose tissue of pigs (described in detail in [7]) were expanded in cell culture for 5-7 days. At passage 3 the cells were simultaneously transfected (using FuGENE 6 Transfection Reagent; Promega Coporation, Madison, WI, USA) with plasmids encoding eGFP fused to the nuclear localization signal H2B and other plasmids containing PiggyBac Transposase (System Bioscience, Mountain View, CA, USA) which was transiently expressed in order to integrate the eGFP cargo into the genome. After transfection, eGFP positive cells were selected for 14 days in complete growth media containing 400 ng/ml G418 (Life Technologies, Carlsbad, CA, USA). Then, cells were separated using fluorescence-activated cell sorting (FACS) using a BD FACSAria Fusion device (BD Bioscience, San Jose, CA, USA). Sorted cells (with >95% of the cells expressing eGFP) were expanded for additional 5-7 days in cell culture. On the day of delivery, eGFP+ cells were trypsinized for 5 min at 37° C, washed twice with PBS, centrifuged at 600 g for 10 min, passed through a 70 μm cell strainer (Falcon, Corning, NY, USA) to avoid cell clumping, and suspended in 10 ml sterile saline solution for delivery (B. Braun Medical Inc., Bethlehem, PA, USA) (on average 10×10^6 cells per animal). For counting cells they were stained with fluorescent nucleic acid stain (SYTO13; Life Technologies, Grand Island, NY, USA) following manufacturer's instructions, and then counted using a hemocytometer under an Eclipse Ti-E inverted fluorescence microscope (Nikon Corporation, Tokyo, Japan) using a PlanFluor 10× objective (numerical aperture [NA] = 0.3) (Nikon). Expression of eGFP was confirmed by fluorescence microscopy during cell counting.

After de-paraffinizing and rehydrating, tissue sections were washed with PBS containing 0.3% Triton X-100 (Sigma Aldrich, St. Louis, MO, USA) and blocked with 10% casein solution (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. Sections were incubated overnight with diluted primary antibodies and subsequently with diluted secondary antibodies for 1 h. The following antibodies were used: Goat anti-GFP, Mouse anti-cardiac troponin T, Rabbit anti-Cx43, Rabbit anti-vWF (all from Abcam, Cambridge, MA, USA); Mouse anti-Ki67, Alexa Fluor 647 conjugated (BD Bioscience); Rabbit anti-goat-IgG secondary antibody, FITC conjugated, Goat anti rabbit-IgG secondary antibody, Cy5 conjugated, Donkey anti-mouse-IgG secondary antibody, TRITC conjugated (all from Life Technologies), and Goat anti-chicken-IgG secondary antibody, Texas red conjugated (Thermo Scientific, Waltham, MA, USA). Counterstaining of nuclei and mounting were performed with Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories).

The photomicrographs shown in this figure were produced by digital photography using a CoolSNAP HQ2 CCD monochrome camera (1392 x 1040 pixels; Photometrics, Tucson, AZ, USA) attached to an Eclipse Ti-E inverted microscope (Nikon) and NIS-Elements AR software (Nikon), using the following objectives (all from Nikon): PlanApo 20× (NA = 0.75) and 40× (NA = 0.95). Merged

figures were constructed using ImageJ software (version 1.51j8; U.S. National Institutes of Health). The final figures were constructed using Corel Photo-Paint X7 and Corel Draw X7 (both versions 17.5.0.907; Corel, Ottawa, Canada). Only minor adjustments of contrast and brightness were made using Corel Photo-Paint, without altering the appearance of the original materials.

5. Summary

This article demonstrates that serious and adequate basic and clinical research is in progress to establish a comprehensive understanding of the potential of UA-ADRCs for regenerative medicine purposes. One of the biggest challenges for the near future is to eliminate the substantial discrepancy between the very high number of publications on "adipose derived stem cells" in PubMed (>10,000; among them approximately 1000 reviews) on the one hand and the very small number of RCTs with UA-ADRCs that have been published so far on the other hand. Authorities worldwide including U.S. FDA will base their judgement about safety and efficacy of tissue regeneration with UA-ADRCs primarily on the results of adequately designed and executed RCTs. As shown here it is indeed possibe to demonstrate safety and efficacy of treatments using UA-ADRCs at the highest possible level of evidence based medicine, to the benefit of the countless patients worldwide who are in need of effective tissue regeneration. We will continue to work consistently to ensure that warnings from authorities about stem cell treatments may one day be a thing of the past.

Author Contributions: Conceptualization, E.A., G.W., C.A. and C.S; methodology, E.A., G.W., C.A. and C.S.; validation, E.A., G.W., A.H., C.A. and C.S.; formal analysis, E.A., G.W., A.H., C.A. and C.S.; investigation, E.A., G.W., A.H., C.A. and C.S.; resources, E.A. and C.S.; data curation, E.A., G.W., A.H., C.A. and C.S.; writing—original draft preparation, C.S.; writing—review and editing, E.A., G.W., A.H., C.A. and C.S.; visualization, C.S.; supervision, E.A.; project administration, E.A and C.S.; funding acquisition, E.A. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: E.A. is Executive Chair of InGeneron, Inc. (Houston, TX, USA). G.W. is employed as Chief Scientific Officer at InGeneron, Inc. C.A. is managing director of InGeneron GmbH (Munich, Germany) which is owned by InGeneron, Inc. C.S. serves as consultant to InGeneron, Inc. A.H. declares no conflict of interest. InGeneron, Inc. and InGeneron, GmbH had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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