Endocannabinoids increase human adipose stem cell differentiation and growth factor secretion in vitro

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Abstract

Adipose stem cells (ASCs) possess the capacity to proliferate, to differentiate into various cell types, and they are able to secrete growth factors. These characteristics are supposed to contribute to their potential for regenerative medicine approaches. In order to advance the therapeutic effects of ASCs, different modulatory procedures have been examined. In this context, the endocannabinoid system (ECS) represents an interesting possibility, since the increased availability of cannabinoids and the underlying molecular pathways of the ECS are of relevance for the development of new regenerative strategies. The effects of the endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) were investigated on ASC metabolic activity, quantified by PrestoBlue conversion, and cell numbers, evaluated by crystal violet staining. enzyme-linked immunosorbent assay (ELISA) measures were performed to determine cytokine release, and differentiation was assessed by specific labeling techniques. AEA increased the metabolic activity, while 2-AG decreased it in a concentration dependent manner. AEA significantly enhanced OilRed O staining after adipogenic differentiation by over 100%, and both compounds significantly increased cresolphthalein staining after osteogenic differentiation. By contrast, they did not affect sphere diameter or safranin O staining after chondrogenic differentiation. Both substances significantly increased the release of insulin-like growth factor-1 and hepatocyte growth factor, while only AEA enhanced transforming growth factor-β secretion. The results demonstrated that stimulating the ECS exerted significant effects on the biology of ASCs. Exposure to endocannabinoids modulated viability, induced release of regenerative growth factors, and promoted adipogenic and osteogenic differentiation. Our findings could be of specific relevance in ASC based therapies for regenerative medicine.

Keywords

adipose stem cell, anandamide, cytokine, differentiation, proliferation, 2-arachidonoylglycerol
INTRODUCTION

Advances in understanding the interaction of biomaterials, growth factors, and progenitor cells contribute to the emerging field of regenerative medicine. In plastic surgery, regenerative medicine promises alternative solutions to classic reconstructive techniques by harnessing the endogenous reparative resources. Indications for such concepts encompass congenital defects, aging or trauma with acute and chronic wounds. Adipose stem cells (ASCs) have been identified suitable for therapeutic application due to their abundance, easy harvest and high regenerative potential (Zuk et al., 2001). These cells have shown an improved outcome in wound healing, as evidenced by their efficacy in case studies of burn wound healing and scarring (Conde-Green et al., 2016), and in pilot studies focusing on ischemic (Lee et al., 2012) and skin cancer-related wounds (Rigotti et al., 2007). Also, phase II randomized clinical trials evidenced the safety of ASC therapy and its positive effects on healing of chronic leg ulcers (Zollino et al., 2019), as well as complex perianal fistulae (Garcia-Olmo et al., 2009).

Although the paracrine activity of ASCs and their ability for multilineage differentiation is unquestioned, the anticipated merits of ASCs have not completely met the initial expectations in the clinical scenario (Patrikoski, Mannerstrom, & Miettinen, 2019). When translating ASCs into clinical practice, the still missing precise comprehension of the ASC biology is one reason for high attrition rates (Arrowsmith & Miller, 2013). To fully exercise their desired regenerative effects at the recipient site, ASCs have to survive, proliferate, release soluble factors or differentiate into distinct cell lineages. In the context of wound healing, several attempts to direct the regenerative capacities and to increase the survival of ASCs have been suggested, including 3-D scaffolds, hypoxia and the supplementation of bioactive proteins (Li & Guo, 2018). Possibilities to modify ASC characteristics in vivo or in vitro are the genetic manipulation or pharmacological conditioning (See, Shin, & Kim, 2019). A new promising approach could target on the endocannabinoid system (ECS). The term ECS derives from the Asian hemp Cannabis sativa, which has been used as medical in ancient times, i.a., for the treatment of wounds (Butrica, 2002). The ECS is an endogenous system that consists of synthesizing and degrading enzymes of the endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and the cannabinoid receptors. It is well-known for its modulatory effects on central nervous system processes, but endocannabinoid signaling is also observed in various nonneuronal tissues, including adipose (Maccarrone et al., 2015).

In a recent study, we investigated the effects of specific activation of the two cannabinoid receptors, CB1 and CB2, of ASCs (Ruhl, Karthaus, Kim, & Beier, 2020). We found that CB2 activation promotes proliferation, while CB1 ligation enhances adipogenesis and chondrogenesis, and agonists of both receptors induce growth factor secretion. The effects of endocannabinoid exposure, which bind to further receptors besides CB1 and CB2, and are thus more comparable to the medical cannabinoids, have not been investigated.

In order to elucidate the role of the ECS in ASC viability, release of soluble factors and differentiation, we exposed ASCs to the endocannabinoids in vitro. Results of this study may unravel potential strategies to:

1. Precondition patients before fat grafting/ASC harvest.
2. Condition ASCs within harvested fat grafts/stromal vascular fraction (SVF) or
3. Condition cultured ASCs for tissue engineering purposes, with the aim to increase the regenerative potential of ASCs.

MATERIAL AND METHODS

2.1 Materials

PrestoBlue, fetal bovine serum (FBS), ITS 1 premix, high glucose medium (4.5 g/L), low glucose medium (1 g/L) and Dulbecco’s Modified Eagle’s medium (DMEM/F-12) were from Life Technologies. Anandamide and 2-Arachidonoylglycerol were bought from Tocris. Collagenase was purchased from Worthington Biochemical Corp. Ascorbate 2-phosphate, β-glycerophosphate, para-formaldehyde (PFA), trypsin–EDTA, o-cresolphthalein complexon, 8-hydroxyquinolinol, penicillin–streptomycin (P/S), 2-amino-2-methyl-1-propanol (AMP), 2-amino-2-methyl-1,3-propanediol (AMPED), Bovine serum albumin (BSA) and TWEEN®20 were obtained from Sigma. L-Prolin, sodium-pyruvate, acetic acid, crystal violet, para-nitrophenylphosphate (pNpp), safranin O, 2 M NaOH, 37% HCl, MgCl₂, were from Roth. Protease inhibitor and insulin was from Roche. Basic fibroblast growth factor (bFGF), transforming growth factor-β3 (TGF-β3) were obtained from PeproTech. Acetic acid, HEPES, isopropyl alcohol, OiIRed O was from Merck. Xylol was from Otto Fischer. Dulbecco’s phosphate buffered saline (PBS) was bought from Biochrom. Phenyl-methylsulfonyl fluoride was obtained from AppliChem.

2.2 Cell culture

Subcutaneous adipose tissue was harvested from n = 15 healthy patients (six male and nine female caucasian patients), with a mean age of 42.93 (SD ± 11.86) years and a mean body mass index of 28.44 ± 3.78 kg/m². The patients negated any prior cannabis medication or use. Adipose tissue was cut en bloc during abdominoplasty and used for ASC isolation as described previously (Ruhl, Storti, & Pallua, 2017). The donors were informed about the cellular use from their tissue and gave informed consent. The study protocol was approved by the regional ethics committee (Ethics Committee of the RWTH Aachen University; EK163/07).

ASCs were expanded in proliferation medium (DMEM with 0.1% bFGF, 10% FBS, 1% P/S), until reaching confluence of ~90%. Experiments were performed with cells from passages two to four seeded at a density of 3 × 10⁵ cells per cm² for experiments targeting viability,
adipogenic and osteogenic differentiation. Adipogenic medium was high glucose medium with 10% FBS, 1% P/S, 10 μg/ml insulin, and chondrogenic medium was low glucose medium with 10% FBS, 1% P/S, 10 ng/ml TGF-β3, 1 μM dexamethasone, 50 mg/ml ascorbate-2-phosphate, 40 mg/ml proline, 100 mg/ml pyruvate, and 50 mg/ml ITS 1 Premix, as described earlier (Ruhl & Beier, 2019; Ruhl et al., 2020). Osteogenic differentiation medium was low glucose medium with 10% FBS, 1% P/S, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, 200 μM ascorbate-2-phosphate. Adipogenic differentiation was analyzed after 7 days, and osteogenic differentiation was performed for 14 days. For chondrogenic differentiation, 10^5 cells were seeded per cm² in 2D-culture or used for pellet culture in eppendorf tubes (Ruhl & Beier, 2019). The cells were exposed to chondrogenic medium for 5 days in 2D or for 21 days in 3D.

### 2.3 Pharmacological stimulation

AEA and 2-AG were dissolved in ethanol and diluted with PBS to yield final concentrations of 0.07% ethanol. PBS containing the respective concentration of ethanol served as vehicle control (Veh). To ensure complete adherence of the cells, cannabinoids were freshly added to the media three days after seeding, except for the 3D chondrogenic differentiation protocol, and during each media exchange.

### 2.4 Crystal violet assay

After seven days, ASCs were stained with 0.1% crystal violet solution as described earlier (Ruhl, Kim, & Beier, 2018). The staining solution was removed and crystal violet was washed out with 33% acetic acid. The samples were transferred to an optical plate and absorbance was quantified at 580 nm in a FLUOstar Optima microplate reader (BMG Labtech).

### 2.5 PrestoBlue assay

Metabolic activity was measured by PrestoBlue (Invitrogen Corporation) following the manufacturer’s instructions. After 1 h of incubation (10% PrestoBlue) at 37°C, 5% CO2 in a humidified atmosphere, 100 μl of the medium were carefully transferred into a 96-well plate and fluorescence was measured in triplets at wavelength of 590 nm (excitation 540 nm).

### 2.6 Growth factor determination by enzyme-linked immunosorbent assay

After seven days of stimulus exposure, the cell supernatant of each well was collected to determine the concentrations of vascular endothelial growth factor (VEGF), the insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), hepatocyte growth factor (HGF), and the transforming growth factor-β1 (TGF-β1) by enzyme-linked immunosorbent assay ELISA Duo-sets (R&D Systems) following the manufacturer’s instructions. Extinction was measured in duplicates per well.

### 2.7 OilRed O staining

Adipogenesis-induced cells were washed in PBS, fixed for 20 min in 4% PFA and stained with OilRed O for 15 min at RT, as described earlier (Ruhl et al., 2017). To determine adipogenesis, adsorbed OilRed O was washed out with 100% isopropanol. Absorbance was measured in triplets at 540 nm.

### 2.8 ALP activity and cresolphthalein staining

For detection of osteogenic differentiation, (I) alkaline phosphatase (ALP) activity or (II) calcium deposition were quantified. (I) Cells were washed with PBS, permeabilized with lysis buffer (1% Tween20 and 1 mM phenylmethanesulfonyl fluoride), and exposed to ALP substrate solution (10 mM pNpp, 100 mM AMPED, and 5 mM MgCl₂, pH = 9.5) for 1 h. The reaction was stopped with 2 M NaOH. Extinction of the supernatant was quantified at 405 nm. (II) Cells were fixed in 4% PFA. After washing with aqua bidest, cresolphthalein-buffer (0.1 mg/ml ortho-cresolphthalein-complexon, 1 mg/ml 8-hydroxychinolin in 30 ml of 37% HCl, diluted in 500 ml aqua bidest) was added and incubated for 5 min. After adding 15% AMP buffer (pH = 10.7) and incubation for 15 min, the extinction of the supernatant was quantified at 580 nm.

### 2.9 Chondrogenic tissue analysis and safranin staining

Chondrogenic spheres from 3D-culture were embedded in tissue freezing medium (Jung, Leica Instruments GmbH), cryosectioned (Leica) into 35 μm slices and stained with Alcian blue using the PAS staining kit (Merck), following the manufacturer’s instructions. Quantification of sphere diameters were performed using the free software ImageJ (Wayne Rasband, Institutes of Health). The chondrogenic monolayers from 2D-culture experiments were stained with safranin O. After 30 min, safranin O was washed out in isopropanol. The samples were transferred to an optical plate and absorbance was quantified at 540 nm.

### 2.10 Analyses and statistics

Data of experiments were grouped and analyzed for normality using the Kolmogorov-Smirnov test. Normally distributed data were expressed as means ± standard error of the mean (SEM),
nonnormally distributed data were presented as box plots. The horizontal black line within each box denotes the median value. The boxes extend from the lower and upper quartiles (defined as the 25th and 75th percentiles) of the data distribution, vertical lines denote adjacent extreme values; according mean values are indicated as dotted lines. Statistical differences were examined between treatments and the Veh. Normally distributed data were tested with the Student’s t-test for unpaired data, nonnormally distributed data were compared using the Mann–Whitney U-test (SPSS 24, SPSS Inc.). Statistical significance was accepted if \( p < 0.05 \).

3 | RESULTS

3.1 | AEA increased and 2-AG decreased metabolic activity

AEA at 1 µM (optical density [OD]_{mean} = 0.54 ± 0.014) and 3 µM (OD_{mean} = 0.53 ± 0.015) had no effect, while cell numbers were significantly reduced at concentrations ≥10 µM (OD_{mean} = 0.47 ± 0.017), when compared to the Veh (OD_{mean} = 0.55 ± 0.016; \( U = 3.34, N_{\text{Veh}} = 28, N_{\text{AEA}} = 30, p = 0.01 \); Figure 1A). On the other hand, the metabolic activity measures of ASCs correlated inversely relative to increasing AEA concentrations. AEA ≥10 µM (OD_{mean} = 8309.9 ± 280.7) significantly increased the PrestoBlue conversion compared to Veh (OD_{mean} = 6829.1 ± 396.3; \( U = 2.58, N_{\text{Veh}} = 28, N_{\text{AEA}} = 30, p = 0.01 \); Figure 1A). The impact of 2-AG was comparable but not identical to the AEA effects (Figure 1B). 2-AG dose-dependently decreased the number of ASCs, with a significant effect at concentrations ≥10 µM (OD_{mean} = 0.525 ± 0.01) compared to Veh (OD_{mean} = 0.56 ± 0.02; \( U = 2.94, N_{\text{Veh}} = 31, N_{\text{2AG}} = 33, p = 0.003 \)). In contrast to AEA, exposure to 2-AG did not increase metabolic activity of the cells but decreased it at concentrations ≥30 µM (\( U = 2.49, N_{\text{Veh}} = 28, N_{\text{2AG}} = 30, p = 0.013 \); Figure 1B). Although the highest levels of metabolic activity were measured at ≥10 µM for AEA, these concentrations significantly reduced the cell numbers. A comparable effect was found for 2-AG. Therefore, both compounds were applied at 3 µM for investigating cell differentiation.

3.2 | AEA and 2-AG increased the release of growth factors

The level of EGF was below the detection limit (data not shown). The vehicle treated cells secreted VEGF at 683 ng/ml (±96 ng/ml),
while exposure to 10 μM AEA increased it to 722 ng/ml (±82 ng/ml) (Figure 2A), but the difference was not significant (t [29] = 1.5; p = 0.13). Exposition to 2-AG decreased the VEGF release without a significant effect. Vehicle treated ASCs secreted TGF-β1 at 3130 ng/ml (±239 ng/ml; Figure 2B), which was significantly increased to 4442 ng/ml (±393 ng/ml) by 10 μM AEA (t [27] = 2.28; p = 0.031), whereas the application of 2-AG did not modulate the release of TGF-β1. ASCs constantly secreted HGF (1319 ng/ml ± 75 ng/ml), which was significantly enhanced by both endocannabinoids (Figure 2C). However, AEA treatment led to higher values of HGF release than found for 2-AG. Concentrations of 2-AG at 1 μM (t [18] = 2.3; p = 0.03) and 3 μM (t [18] = 2.6; p = 0.02) significantly increased the HGF secretion, but not at 10 μM (t [18] = 0.2; p = 0.8; Figure 2C). The IGF-1 release was at 12 pg/ml in Veh treated ASCs (Figure 2D), but both endocannabinoids increased the IGF-1 levels in a dose dependent manner. AEA had a significant effect on IGF-1 release at 10 μM (t [21] = 3.0; p = 0.007), while a significant 2-AG impact was measured at 3 μM (t [21] = 2.5; p = 0.02; Figure 2D).

### 3.3 | AEA promoted adipogenic differentiation

For examination of adipogenesis, ASCs were cultured in proliferation medium (Figure 3A) or in adipogenic differentiation medium containing insulin (Figure 3B). Basal levels of OilRed O staining were higher when the cells were cultured in differentiation medium (OD\text{mean} = 0.075 ± 0.003) than in proliferation medium (OD\text{mean} = 0.036 ± 0.004). In both approaches, exposure to 3 μM AEA significantly increased lipid staining (U = 4.6, N\text{Veh} = 42, N\text{AEA} = 24, p < 0.001; U = 4.5, N\text{Veh} = 79, N\text{AEA} = 50, p < 0.001) by about 100%, while 2-AG did not produce an effect (Figure 3A–B).

### 3.4 | AEA and 2-AG promoted osteogenic differentiation

While 2-AG had no impact on ALP activity (OD\text{mean} = 2.1 ± 0.16), AEA (OD\text{mean} = 2.4 ± 0.19) significantly increased it (U = 2.7, N\text{Veh} = 33, N\text{AEA} = 35, p = 0.006) over the Veh (OD\text{mean} = 1.8 ± 0.12; Figure 4A).
In accordance with the increase of this early osteogenesis marker, the calcium content of the extracellular matrix of the osteogenically stimulated ASCs under exposure to 3 μM AEA (OD\textsubscript{mean} = 0.33 ± 0.006) was significantly increased (U = 3.4, N\textsubscript{Veh} = 33, N\textsubscript{AEA} = 36, p < 0.001) compared to Veh (OD\textsubscript{mean} = 0.29 ± 0.01). In contrast to ALP activity, 2-AG (OD\textsubscript{mean} = 0.33 ± 0.005) significantly increased calcification over Veh (U = 3.1, N\textsubscript{Veh} = N\textsubscript{2-AG} = 33, p < 0.001; Figure 4B).

3.5 | Endocannabinoids did not affect chondrogenic differentiation

Chondrogenic differentiation was assessed by measuring the diameter of chondrogenic spheres (Figure 5A) or by safranin O staining of cell monolayers (Figure 5B). At the end of the 3D-culture experiments, the cells were organized in round-shaped spheres, and chondrogenic differentiation could successfully be determined by Alcian blue staining (Figure 5A). Treatment with chondrogenic differentiation medium generated spheres of 1038 μm (±19 μm) diameter on average. Larger diameters were measured for 3 μM AEA (1048 ± 15 μm) and for 3 μM 2-AG (1051 ± 17 μm), but the increases were not significantly different from Veh (Figure 5A). Similar findings were detected by safranin O staining. Vehicle treated cells were stained at OD\textsubscript{mean} = 1.53 (±0.07), which was not significantly different from the cells exposed to AEA (OD\textsubscript{mean} = 1.63 ± 0.1) or the ASCs treated with 2-AG (OD\textsubscript{mean} = 1.8 ± 0.1) (Figure 5B).

4 | DISCUSSION

4.1 | Endocannabinoids regulate viability of ASCs

Since the detection of the cannabinoid receptors on the plasma membranes of ASCs, their influence on cellular vitality has been
investigated using exocannabinoids. Most of these studies were performed with rodent cells and showed that blocking the CB1 receptor inhibits proliferation (Bellochio, Cervino, Vicennati, Pasquali, & Pagotto, 2008), which could not be observed in human ASCs (Ruhl et al., 2020). By contrast, we found that both AEA and 2-AG decreased cell numbers at increasing concentrations. Anti-proliferative effects and cell death mechanisms through cannabinoids have been observed before, but mostly in different cancer cells (Calvaruso, Pellerito, Notaro, & Giuliano, 2012). Since the cell count has been performed only once at the end of our experiment, the rate of the cell growth cannot be seen. This would have been important in order to differentiate between a cytotoxic and an anti-proliferative effect of endocannabinoid exposure. Thus, an additional, independent method to exclude cytotoxicity is recommended, for example, G6PD- or LDH-release assay from the supernatants.

We found that specific activation of CB1 decreases, whereas ligation to CB2 increases cell numbers (Ruhl et al., 2020). Since both endocannabinoids show affinity to CB1 as well as to CB2, it is possible that at increasing concentrations their activity at CB1 superimposed their CB2 effects, which in turn decreased cell numbers. Furthermore, although both endocannabinoids inhibited ASC growth, AEA increased but 2-AG decreased the metabolic activity. This surprises since both ligands bind to CB1, and it has been described that CB1 activation increases intracytoplasmic cAMP as a molecular signal involved in regulation of energy homeostasis (Ravnskjaer, Madiraju, & Montminy, 2016). Thus, the dissimilar effects of both receptor ligands could be based on varying binding affinities to CB1 but also to non-CB receptors. AEA is considered a high affinity agonist with high CB1 selectivity, whereas 2-AG is a moderate affinity CB1 and CB2 agonist (Di Marzo & De Petrocelli, 2012). Furthermore, AEA but not 2-AG binds to the transient receptor potential of vanilloid type-1 (TRPV1) channels (Di Marzo & De Petrocelli, 2012; Pertwee et al., 2010), which is a nonselective cation channel that guides calcium ions into the cells (Yang & Zheng, 2017). These binding characteristics could contribute to the varying findings between the two ligands.

Alternatively, the different routes of AEA and 2-AG degradation and metabolism might explain our findings: The predominant pathway for 2-AG metabolism is the hydrolysis of the ester bond into arachidonic acid and glycerol (Baggelaar, Maccarrone, & van der Stelt, 2018). Arachidonic acid is part of the innate immune response and it induces or suppresses inflammation (Bennett & Gilroy, 2016). Glycerol is regularly secreted by adipose tissue as a by-product of
lipolysis. The capacity of adipocytes to recycle glycerol is limited, but glycerol is a main substrate for hepatic gluconeogenesis (Rotondo et al., 2017). Fatty acid amide hydrolase also catalyzes the hydrolysis of 2-AG, but it is mainly responsible for the cleavage of AEA into arachidonic acid and ethanolamine (Maccarrone, 2017). The latter one can be converted into acetyl-CoA, acetate and ATP, which can be used in a variety of metabolic processes and would account for our findings on the increased PrestoBlue conversion (Zhou et al., 2017).

4.2 | Endocannabinoids modulate cytokine release of ASCs

Activation of CB1 and CB2 by specific receptor ligands induces the release of growth factors on a comparable level as found for endocannabinoids in the present study (Ruhl et al., 2020). HGF regulates cell growth and mobilization of several cell types, including epithelial and endothelial cells, confirming its contribution to epithelial repair and neovascularization (Conway, Price, Harding, & Jiang, 2006). Imbalances in activation and deactivation of the HGF pathway are important pathogenetic factors in chronic wounds (Behm, Babilas, Landthaler, & Schreml, 2012). Therefore, the topical application of HGF or its increase through ECS activation on secreting cells could be a potential therapy in wound healing. IGF-1 is wound induced in animals and humans (Gartner, Benson, & Caldwell, 1992). IGF-1 promotes wound healing by multiple mechanisms, while its levels are decreased in wounds with low regenerative potential, such as diabetic wounds (Blaktyny & Jude, 2006). There are many studies indicating that the addition of exogenous IGF-1 accelerates wound healing in rodent animal models, principally by dampening the local inflammatory response and promoting re-epithelialization (Emmerson et al., 2012). Thus, an increased release of IGF-1 by ECS stimulated ASCs could be of benefit for cell driven therapy in wound healing. Admittedly, we tested the highest levels of endocannabinoid exposure, which did not reduce cell numbers. It is very unlikely that these concentrations correspond to the physiologic concentrations under in vivo conditions. Thus, also minor concentrations in the submicromolar range should be tested in future experiments.

FIGURE 5  Influence of AEA (red) and 2-AG (green) on chondrogenic differentiation of ASCs. The cells (3 donors) were cultured in differentiating medium supplemented with either vehicle (Veh) or endocannabinoid ligands at 3 µM. (A) After 21 days of incubation, chondrogenic spheres were cryo-sectioned and stained with Alcian blue for determination of sphere diameters (n = 12–18). (B) After 5 days of exposure, proteoglycan formation was quantified by OD of safranin O staining (n = 32–34). Box plots represent medians calculated from optical densities, dotted lines indicate mean values; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; ASC, Adipose stem cell; OD, optical density; Veh, vehicle control [Colour figure can be viewed at wileyonlinelibrary.com]
4.3 | Endocannabinoids increase differentiation capabilities of ASCs

Exposure to the exocannabinoids WIN55,212-2 and Δ⁹-THC as well as to AEA increases adipogenic differentiation in murine cells (Belloccchio et al., 2008; Teixeira et al., 2010). The amplification in adipogenesis is accompanied by an up-regulation of the endocannabinoid receptor CB1 and of PPAR-γ, which indicates the associated receptor participation (Karaliot, Siafaka-Kapadai, Gontinou, Psarria, & Mavri-Vavayanni, 2009). In a recent study on human ASCs, we confirmed these earlier findings, namely that ligation to the CB1 induces adipogenic differentiation (Ruhl et al., 2020). Another study on human MSCs reported that AEA promotes adipogenesis, while 2-AG has no such effect (Ahn et al., 2015). This is in accordance with our findings on ASCs. AEA induced adipogenesis when the cells were kept in proliferation medium, and it promoted adipogenic differentiation at co-exposure with insulin as initiator of adipogenesis. Cannabinoids increase glucose uptake via CB1 in ASCs, which introduces them as insulin-mimetic substances (Gasperi et al., 2007). Since AEA is considered a high affinity agonist with strong CB1 selectivity, whereas 2-AG is a moderate affinity CB1 and CB2 agonist (Di Marzo & De Petrocellis, 2012), these binding characteristics could explain the varying findings between both ligands.

All key components of the ECS were found in bone tissue, and AEA and 2-AG are present in this tissue at levels similar to those found in the brain (Bab, Ofek, Tam, Rehnelt, & Zimmer, 2008). This indicates a fundamental role of the ECS in bone formation. In the present study, AEA and 2-AG increased the level of calcium deposition. We could recently show that activating the CB2 receptor increases osteogenic differentiation in ASCs (Ruhl et al., 2020), which represents a target for both endocannabinoids. However, only AEA increased the enzyme activity of ALP, which is important for the mineralization of bone cells and tissue. This suggests that 2-AG affected osteogenesis differently from AEA. The role of the ECS in chondrogenesis has been less well investigated. The treatment of rat MSCs with Δ⁹-THC enhances chondrogenic differentiation by increasing the expression of collagen and proteoglycan (Gowran et al., 2010). In ASCs, the exocannabinoid WIN55, 212-2 increases chondrogenic differentiation (Ruhl et al., 2020), but endocannabinoids did not exert an effect in the present study. It is possible that the absence of any effect could reflect ligand interactions with non-CB receptors, which inhibited their chondrogenic activity.

5 | CONCLUSION

ASCs represent a cell population that holds great promise for regenerative medicine. Although there is a deficit in number of randomized controlled trials and quantitative analysis supporting the efficacy of ASCs, their therapeutic effects in regenerative medicine are promising. Furthermore, these cells express an ECS, which is ascribed significance in tissue regeneration (Wang et al., 2016).

The present study suggests the ECS as a novel candidate for enhancing the regenerative capacity of ASCs, including applications in wound repair. Although more precise assays are required to make conclusive statements on their value on wound healing, for example, ASC migration in scratch assay or co-culture with keratinocytes. AEA and 2-AG increased the release of growth factors and promoted their differentiation capabilities. Translated into the clinical situation, a chronic low dose of medical cannabis applied to a patient prior fat grafting may be a possible way to modulate ASCs for regenerative approaches (Figure 6, green arrows). Alternatively, in vitro cultured ASCs may be stimulated by cannabinoids and injected in a second procedure (Figure 6, red arrow). Approved cannabinoid substances are Sativex, the medicinal THC that is an under-the-tongue spray, as well as its synthetic forms, that is, nabilone, dronabinol, marinol, and Syndros. By interacting with the ECS, they are effective in pain patients who are resistant to other pharmacological interventions, like in rheumatoid arthritis pain, cancer pain and also in central and peripheral neuropathic (Russo, 2008; Tanasescu & Constantinescu, 2013). Furthermore, they are used in the treatment of chemotherapy induced nausea and vomiting (Gerrn et al., 2010). A possible effect of these substances on ASCs in vivo has not been experimentally investigated. Therefore, the next experimental step would be an animal study to investigate the effect of continuous ECS stimulation on ASC parameters after isolation.

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