Skin Pharmacology and Physiology

Autologous Cell Therapy for Aged Human Skin: A Randomized, Placebo-Controlled, Phase-I Study

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Abstract

Introduction: Skin ageing involves senescent fibroblast accumulation, disturbance in extracellular matrix (ECM) homeostasis, and decreased collagen synthesis. *Objective:* to assess a cell therapy product for aged skin (RCS-01; verum) consisting of ~25×10^6 cultured, autologous cells derived from anagen hair follicle non-bulbar dermal sheath (NBDS). *Methods:* For each subject in the verum group, 4 areas of buttock skin were injected intradermally 1 or 3 times at monthly intervals with RCS-01, cryomedium, or needle penetration without injection; in the placebo group RCS-01 was replaced by cryomedium. The primary endpoint was assessment of local adverse event profiles. As secondary endpoints, expression of genes related to ECM homeostasis was assessed in biopsies from randomly selected volunteers in the RCS-01 group taken 4 weeks after the last injection. *Results:* Injections were well tolerated with no severe ad- verse events reported 1 year after the first injection. When compared with placebo-treated skin, a single treatment with RCS-01 resulted in a significant upregulation of TGFβ1, CTGF, COL1A1, COL1A2, COL3A1, and lumican mRNA expression. *Limitations:* The cohort size was insufficient for dose ranging evaluation and subgroup analyses of efficacy. *Conclusions:* RCS-01 therapy is well tolerated and associated with a gene expression response consistent with an improvement of ECM homeostasis.

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Introduction

There is growing evidence that ageing of human skin is driven by the senescence of fibroblasts located in the upper dermis. Cells expressing markers of senescence are present at increased frequencies in aged skin of primates and humans [1, 2]. Primary skin fibroblasts isolated from intrinsically aged human skin exhibit molecular hall-

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marks of cellular senescence, including a secretory factor profile which is indicative of incipient cell senescence [3]. This secretory phenotype of ~70 proteins, secreted in an age-dependent manner, are collectively termed the skin ageing-associated secretory phenotype (SAASP). Functional annotation analysis revealed that these proteins can be assigned to 14 different biological processes, among which extracellular matrix (ECM) organization was the most prominent. A detailed enrichment and network analysis using the "Reactome database" revealed that "ECM organization," "elastic fiber formation," "activation of matrix metalloproteinases" and "collagen degradation" categories had the strongest association with intrinsic skin ageing. These 4 biological processes are closely linked to ECM remodelling in general, and are of obvious relevance for defining the clinical, histological, and molecular features of aged human skin. They are linked to a profound disturbance in collagen homeostasis because of a decrease in de novo collagen synthesis and an increase in collagen degradation [4]. The secretion of these proteins by senescent fibroblasts may be of pathogenetic relevance for the ageing process of human skin and, potentially, modulation of this secretion pattern might alter skin ageing.

The emerging field of regenerative medicine focuses on stem cell, progenitor cell, and tissue-based therapies. It involves exploitation of cells and their proliferative capacity, potential to differentiate, paracrine signalling activity, and because they can be sourced autologously avoiding implant rejection or the need for immunosuppressive therapy [5].

To this end, we developed a cell-therapeutic approach based on the intradermal injection of autologous, cultured mesenchymal cells, isolated from the non-bulbar dermal sheath (NBDS) of hair follicles. The NBDS represents a unique source of fibroblasts, which express several proteins that potentially support ECM maintenance, most notably type 1 collagen [6–8]. Cultured NBDS cells provide a promising platform to treat intrinsically or extrinsically aged/damaged skin such as fine wrinkles by implanting UV naïve, collagen-producing NBDS cells directly to the affected area. As a first clinical step, we conducted a first-in-human clinical trial to determine, as a primary objective, the local safety profile of this cell-therapeutic treatment, as defined by the incidence, causality, severity and seriousness of adverse events (AEs). In addition, as a secondary objective, we analysed if and how this treatment affects gene expression in human skin. We focused on genes which were previously found to be associated with skin ageing and linked to proteins, which are part of the SAASP. We hypothesised that injecting autologous, cultured NBDS cells would be safe and would modify the local human skin gene expression profile consistent with increased collagen production and a reduced SAASP profile.

Materials and Methods

Study Design and Subjects

This clinical study was conducted at the IUF – Leibniz Research Institute for Environmental Medicine (Düsseldorf, Germany). It was approved by the Ethics Committee of the Heinrich Heine University (Düsseldorf, Germany), and received clearance by the Paul Ehrlich Institute competent authority for cell therapy in Germany. The study design was a phase-I, randomized, double-blind, placebo-controlled, single-centre study. Inclusion and exclusion criteria were as described (online suppl. Table SI; for all online suppl. material, see www.karger.com/doi/10.1159/000502240). A total of 26 subjects were screened, of which 21 were randomized and 17 completed the study protocol (demographic data in online suppl. Table SII).

Treatments

The study was initiated on October 6, 2015, and the last study visit was conducted on May 16, 2017. The study included a total of 14 visits, that is, 3 screening visits and 11 visits during the treatment and observation period. At visits 1, 3, and 5 (Fig. 1a), the study subjects were injected intradermally with a cell therapy product (RCS-01; verum) manufactured from the subject's own (autologous) NBDS cells isolated from hair follicles, or they received the respective placebo. For this, a punch biopsy was taken at the IUF from the back of the subject's scalp (occiput) at the second screening visit (visit S2; Fig. 1a). The tissue sample was then transferred to biopsy media and subsequently shipped to the cGMP manufacturer of the RCS-01 product (Innovacell Biotechnologie AG, Innsbruck, Austria). At the manufacturing site, the tissue biopsy was dissected to isolate hair follicle NBDS, which were then put into culture, during which the NBDS cells were replicated to achieve 25×10^6 ($\pm 3 \times 10^6$) NBDS cells per individual batch. The NBDS cells were then suspended in cryomedium composed of Ringer's lactate containing 2% human serum albumin and 5% DMSO. RCS-01, as well as placebo, were supplied by the manufacturer in coded, single-dose vials. Placebo vials contained cryomedium only. Vials containing RCS-01 or placebo were then shipped from the manufacturing site to the study site and kept below –130 °C until administration. At the study site, verum and placebo products were injected in a volume of 1.0 mL evenly distributed in 6 injections throughout the approximately 2-cm^2 area. A syringe holder controlled the depth, angle, and volume of the injections into the dermis of the buttock. Sham injections involved only needle penetration with no fluid injected (Fig. 1, 2).

Randomization and Masking

Randomization of subjects to the 2 treatment groups (RCS-01 group and placebo group) in a 4:1 ratio and the 2 post-treatment biopsy groups (visit 7 biopsy group and visit 9 biopsy group; Fig. 1b) used 2 separate randomization lists, which were computer generated by the Pharmalog Institut für Klinische Forschung GmbH (Munich, Germany), which served as the Clinical Research Organization (CRO) in this study. The vehicle-treated area in the
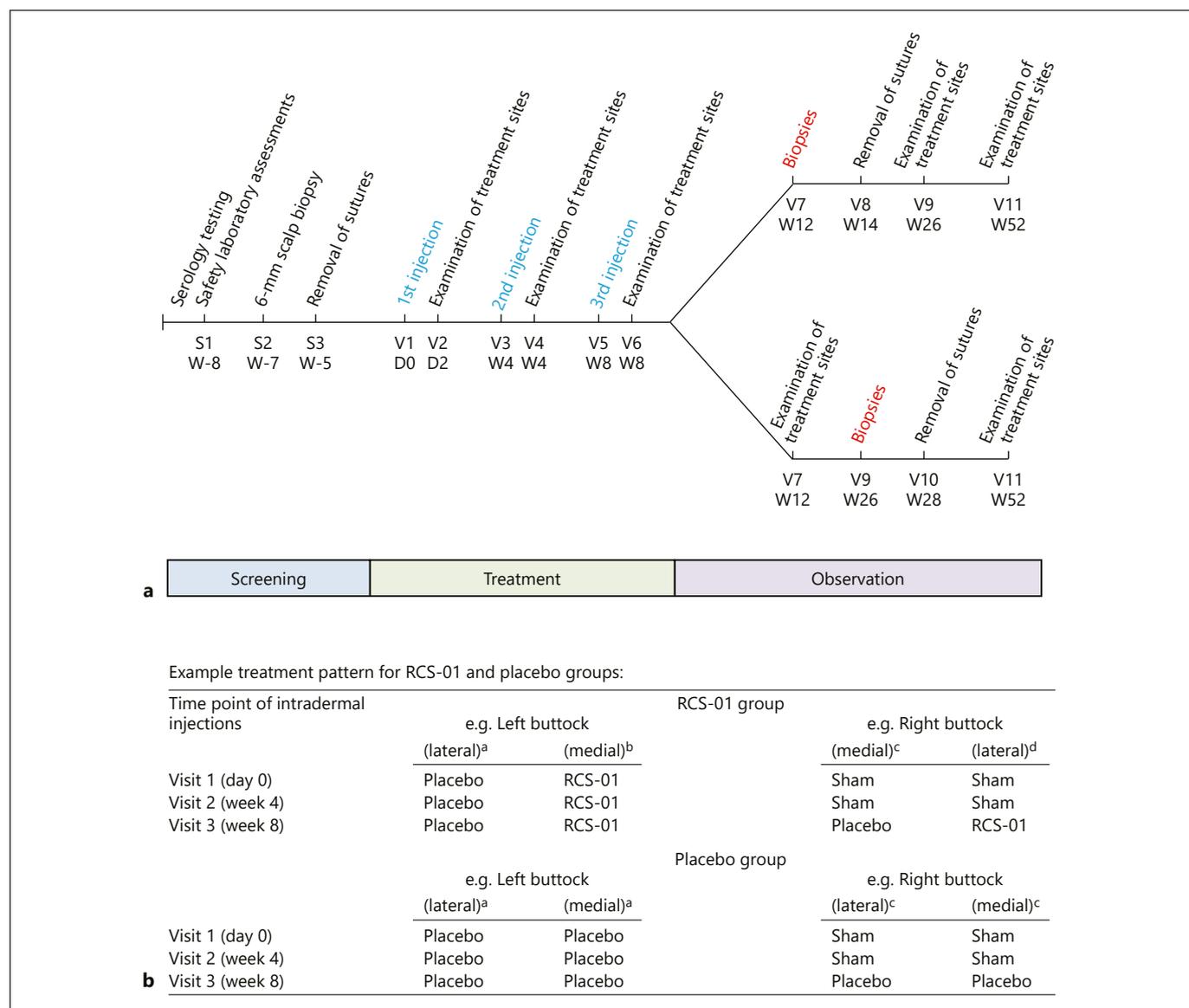
Fig. 1. A summarized schedule of assessments (**a**) and an example treatment pattern for the RCS-01 and placebo treatment group (**b**). Each subject had 4 treatment sites on the buttock (2 on each buttock), which were identified by a tattoo. On visit 1, subjects from the RCS treatment group received either placebo (cryomedium), RCS-01 (human autologous cultured hair follicle NBDS cells in 1-mL cryomedium), or sham injections (needle penetration without injection of any fluid) according to a randomization schedule. After 4 weeks, the subjects returned and received the same treatment in the same pattern as before. Treatment evaluation sites treated with 2 doses of placebo or RCS-01 at visits 1 and 3 received a third injection of RCS-01 or placebo. The other 2 treatment evaluation sites previously receiving sham injections randomly received injections of either RCS-01 or placebo. For subjects in the placebo group, the treatment pattern was similar except that RCS-01 was replaced by placebo. The superscript letters indicate the following: [a] triple treatment placebo (placebo/placebo/placebo); [b] triple treatment verum (RCS-01/RCS-01/RCS-01); [c] single treatment placebo (sham/sham/placebo); [d] single treatment verum (sham/sham/RCS-01).

group of RCS-01-receiving volunteers served as an intraindividual control for evaluation of local AEs compared to the verum-treated area in the same volunteer, while the smaller group only receiving placebo served as a control for systemic AEs.

The randomization ratio used for allocation to one of the injection sites of the 4 (RCS-group)/2 (placebo group) treatment patterns was 1:1:1:1 and 1:1, respectively (Fig. 2). As RCS-01 and placebo were visually different, an unblinded study team member not involved in any post-administration evaluations performed all injections, including sham injections, at visits 1, 3, and 5. Related information was kept separate from other records and was accessible only to the unblinded study team member.
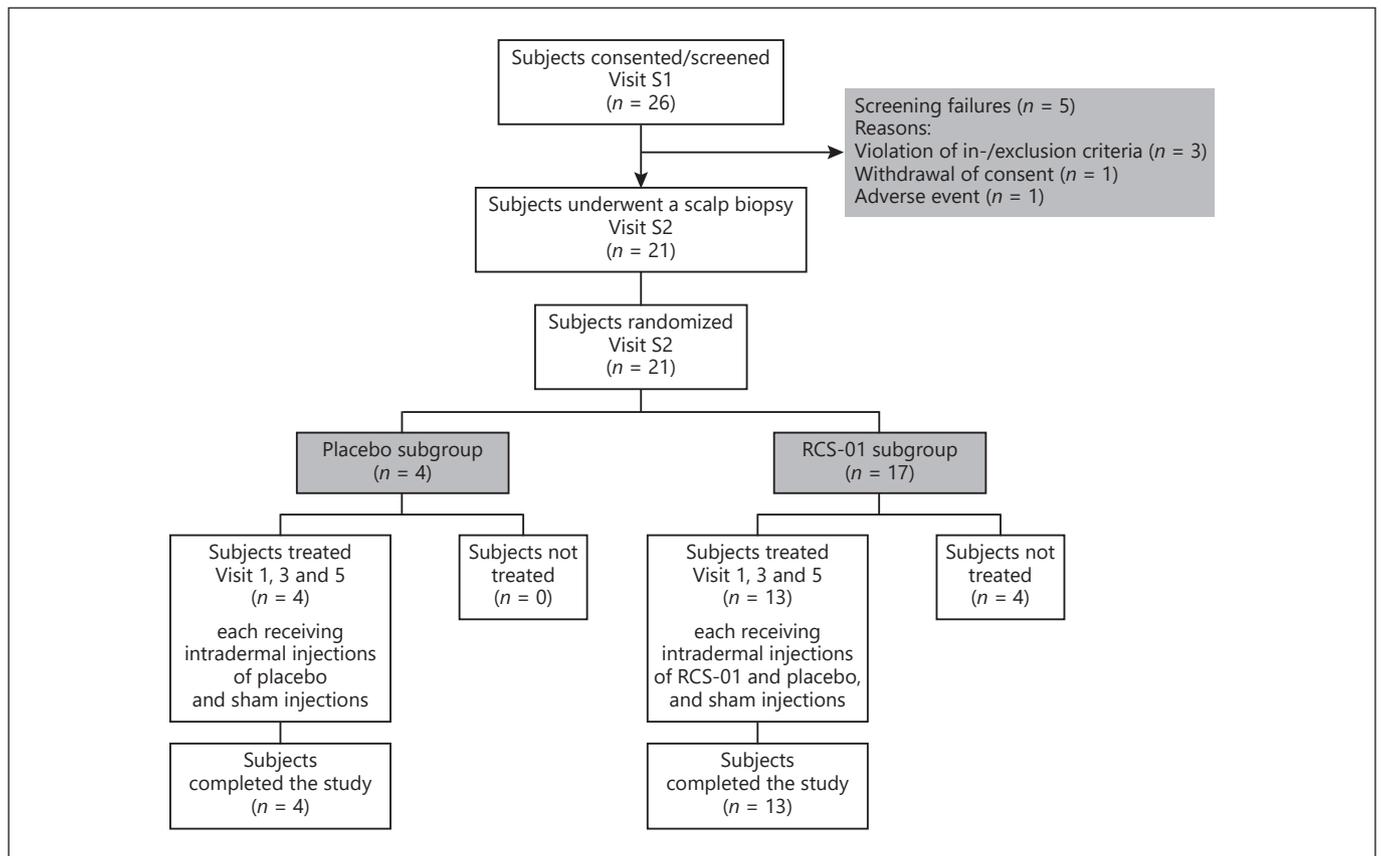
Fig. 2. Selection and randomization of trial subjects. RCS-01, human autologous cultured hair follicle NBDS cells in 1-mL cryomedium; placebo, cryomedium; sham injection, needle penetration of the skin without injection of fluid.

Safety Parameters

Clinical safety assessments included height, body weight, vital signs, an abbreviated clinical examination, serology laboratory assessments according to the exclusion criteria, safety laboratory assessments including haematology (RBC, haematocrit, MCV, MCHC, reticulocytes, WBC with differential, platelet count), biochemistry (sodium, potassium, creatinine, total protein, AP, total cholesterol, ALT, AST, BUN, CRP), and dipstick urinalysis (protein, glucose, ketones, bilirubin, blood, urobilinogen, pH, specific gravity, nitrite, haemoglobin, leukocytes), investigator assessment of the treatment evaluation sites for local intolerance (solicited local AEs: erythema, bruising/haemorrhage, pyoderma/infection, eczema, granuloma/papules/nodules, hypertrichosis/hair density, hypopigmentation, hyperpigmentation, scar), and AEs. A detailed definition of the AEs is given in Table 1. In addition, histopathological evaluation of treatment sites was performed on biopsies obtained at visit 9 (Fig. 1a).

Efficacy Parameters

Gene expression studies used total RNA which was extracted from punch biopsies obtained from all 4 treatment sites at visit 7 (Fig. 1a) as previously described [9–12]. Frozen biopsies were disrupted in lysis buffer from an RNA isolation kit (Peq-Gold Total RNA Kit (Peqlab, Erlangen, Germany) using a Mixer Mill MM300 (Retsch, Haan, Germany) 3 times for 3 min with 30 Hz. Isolated RNA was photometrically quantified and 50 ng of total RNA was used for cDNA synthesis with M-MLV RT (Life Technologies, Karlsruhe, Germany). An aliquot was used for pipetting PCR reactions with ABsolute QPCR SYBR Green Mix (Thermo Fisher Scientific, St. Leon, Germany) with the help of an epMotion 5070 pipetting device (Eppendorf, Wesseling-Berzdorf, Germany). PCR reactions, using primer sequences as described (online suppl. Table SIII), were performed in a CFX384 Real-Time PCR Detection System (Bio-Rad Laboratories GmbH, Munich, Germany) after 15 min at 94°C for activation of hot-start Taq polymerase. Cycles consisted of 20 s of denaturation (95°C), 20 s of annealing (55°C), and 20 s of extension (72°C). For comparison of relative gene expression in real-time PCR the $2(-\Delta\Delta CT)$ method was used [13].

Pro-Collagen ELISA Assay

Cultures of NBDS cells were examined for the expression of pro-collagen ($n = 5$). Briefly, NBDS cells were cultured using the clinical trial protocols. Cells were plated post-collection at the end of passage 4 of primary culture (8×10^4 cells per well in a 6-well plate; Corning, Tewksbury, MA, USA) in duplicates, equivalent to the point at which cells are injected in the clinical trial. Culture supernatants were sampled at 0, 3, and 6 h post-plating, and the

Table 1. Overview of subjects with AE

Systemic/local AEs	RCS-01 subgroup (n = 13)	Placebo subgroup (n = 4)	Total (n = 17)
Total subjects with AEs	10 (76.9)	4 (100.0)	14 (82.4)
Subjects with at least 1 local AE	10 (76.9)	4 (100.0)	14 (82.4)
Solicited local AEs	10 (76.9)	4 (100.0)	14 (82.4)
Unsolicited local AE	0	0	0
Subjects with at least 1 systemic AE	3 (23.1)	1 (25.0)	4 (23.5)
Onset before first treatment (visit S1 – visit 1)	2 (15.4)	0	2 (11.8)
Onset after first treatment (visit 1 – visit 11/12)	1 (7.7)	1 (25.0)	2 (11.8)
Subjects with at least 1 serious AE	0	0	0

Data are presented as *n* (%). AE, adverse event.

concentration of pro-collagen type I was quantified according to the manufacturer's instructions (Procollagen Type I C-Peptide EIA Kit; Takara-Bio, Mountain View, CA, USA).

Statistical Analysis of Gene Expression

Data are given as x-fold induction compared to a placebo control as median (25–75% percentiles). Statistical evaluation and graphical presentation were performed with SigmaPlot 12.3 (Systat Software GmbH, Erkrath, Germany). Normality of the data was tested using the Shapiro-Wilk test. For comparison of significant differences, Kruskal-Wallis one-way analysis of variance on ranks was employed. As post hoc analysis, the Student-Newman-Keuls test was used. A probability level of $p < 0.05$ was considered significant. Data are presented as box plots with median and corresponding percentiles.

Results

A total of 26 subjects (online suppl. Table SII) were screened at screening visit 1. From these, 5 were not randomized due to the presence of exclusion criteria (*n* = 3), withdrawal of consent (*n* = 1), or onset of an AE (*n* = 1) that prevented the subject returning for screening visit 2. The remaining 21 subjects had a scalp biopsy and were randomized to the 2 treatment groups at screening visit 2 (Fig. 2). From these, 4 subjects, who had been randomized to the verum treatment group, did not receive any intradermal injections of study products because NBDS cell cultivation was not successful due to poor biopsy quality.

Of the 17 subjects (15 females and 2 males, mean age of 55.1 years) who completed the study, 13 (76.5%) were in the RCS-01 group (Fig. 2), that is, they received intradermal injections of RCS-01 and placebo and sham injections, whereas 4 subjects (23.5%) were in the placebo group (Fig. 2), that is, they received intradermal injec-

tions of placebo and sham injections, but not RCS-01 injections. This approximately reflects the intended ratio of 4:1. All 17 subjects also completed the 44-week post-injection follow-up period.

The safety data of the 13 individuals in the RCS-01 subgroup demonstrated a good local and systemic safety profile of RCS-01 injections up to 1 year after single and triple intradermal injections. Serious local or systemic AEs did not occur. There was no evidence of systemic toxicity. No clinically relevant changes were observed in mean values of systolic/diastolic blood pressure, pulse rate, body temperature, and body weight of the treated subjects. There were neither clinically significant abnormal laboratory test results shortly after study treatment nor any clinically relevant changes during the course of the study. Fourteen out of 17 treated subjects reported at least 1 local and/or systemic AE (Table 1). One out of 13 subjects treated in the RCS-01 group and 1 out of 4 subjects treated in the placebo group experienced at least 1 systemic AE after the first treatment (treatment-emergent AEs). All systemic AEs were considered unrelated or unlikely related to the study treatment and had resolved by the end of the observation period.

Ten out of 13 subjects in the RCS-01 group, and all 4 subjects in the placebo group, presented with at least one solicited local AE of mild to moderate intensity, which were all transient in nature. Mild bruising (haemorrhage) at the injection site was the most common local AE, with a similar occurrence after intradermal administration of RCS-01 and placebo and sham injections.

Repeated intradermal injection of RCS-01 was associated with a higher occurrence of granuloma, papules, or nodules compared to control injection of cryomedium (placebo). Hyperpigmentation, eczema, and ery-
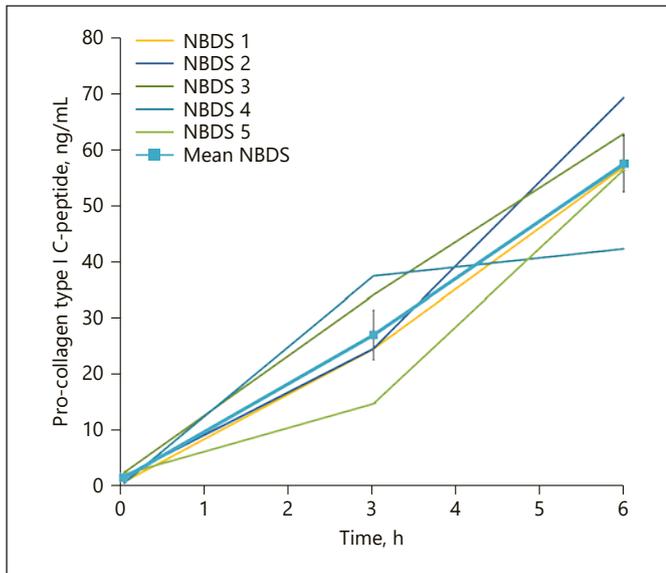
Fig. 3. Concentration of pro-collagen type I C-peptide in culture supernatants from 8×10^4 non-bulbar dermal sheath cells (NBDS) processed and cultured from 5 individuals, and mean concentration for NBDS cells.
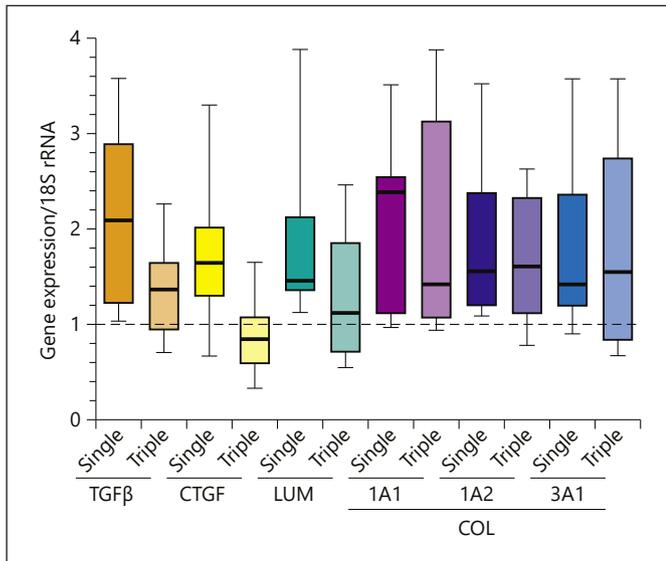
Fig. 4. Upregulation of mRNA levels for dermal markers after single or triple treatment with RCS01 compared to a single placebo treatment. Given are box plots of $n = 9$; error bars reflect 95 and 5% percentiles. Dashed line indicates placebo-treated level set arbitrarily equal to 1. ANOVA on ranks versus first single placebo treatment.

thema were observed rarely and showed no increased occurrence after repeated treatment with RCS-01 and placebo. There were no signs of pyoderma (infection), hypertrichosis, and hypopigmentation at the injection sites of RCS-01 or placebo. In addition, histopathological assessment of 32 treatment sites from 8 subjects, who were biopsied 4 months after the last injection, revealed no abnormalities or structural changes (data not shown).

In terms of pro-collagen type I C-peptide expression post-production (after passage 4), NBDS cells exhibited relatively consistent levels of pro-collagen expression (mean at 6 h, 57 ng/mL/8×10^4 cells; range 42–69 ng) in culture supernatants (Fig. 3).

Gene expression analysis was performed using biopsies obtained from 7 subjects of the RCS-01 group at visit 7 (Fig. 1a), that is, 1 month after the last injections with RCS-01 and placebo. A single injection of RCS-01 caused a significant increase in mRNA expression of all genes, as compared with mRNA expression of these genes in placebo-treated skin (online suppl. Table SIV). Notably, although we did not assess the impact on clinical signs of ageing such as wrinkle formation, some of the strongest responses were observed for TGFβ1, and also for genes involved in de novo collagen synthesis such as COL1A1, COL1A2, and COL3A1 (Fig. 4). Increased gene expression was also observed when RCS-01 was injected 3 times, but overall the response was weaker than that observed after single treatments and mainly included COL1A1, COL1A2, and COL3A1.

Discussion/Conclusion

In this study we show for the first time that single and repeated injections of NBDS-derived autologous cells into the dermis of human buttock skin are well tolerated and do not cause systemic or local adverse reactions for a period of up to 1 year after the first injection. We also provide evidence that this cell-therapeutic approach alters the transcriptional expression profile of genes which are involved in ECM homeostasis. Specifically, we have found that in comparison to placebo-treated skin areas, injection of NBDS-derived fibroblasts induces the expression of TGFβ1, CTGF, COL1A1, COL1A2, COL3A1, and lumican.

These genes encode for proteins which are important for ECM homeostasis, thought to be of pathogenetic relevance for skin ageing, where levels of types I

and III collagen precursors and crosslinks are reduced [14]. Accordingly, TGFβ1 is the major profibrotic cytokine and, together with CTGF, synergistically stimulates type 1 procollagen synthesis in adult human fibroblasts [15]. Also, COL1A1 and COL1A2 are crucial for de novo synthesis of type 1 collagen chains, which are markedly decreased in aged human skin [14–16]. Lumican belongs to the small leucine-rich proteoglycan family and is involved in collagen fibril formation in skin [17]. Upon UV irradiation, lumican expression is decreased in human dermal fibroblasts, which might contribute to the downregulation of pro-collagen I in UV-irradiated skin [18].

The present observation that injection of NBDS-derived cells is associated with an increased transcriptional expression of these genes indicates the possibility that cell therapy with RCS-01 might be able to ameliorate the clinical signs of skin ageing, such as wrinkles. This assumption is in line with observations that increased expression of collagen types 1 and 3 is a prerequisite for wrinkle reduction caused by retinol [19]. We therefore believe that our observations warrant corresponding phase-2 studies to directly assess this possibility. Insufficient cohort size for dose ranging evaluation and subgroup analyses of efficacy was the primary limitation of the current study. Future clinical studies will explore dose responses, as well as the possibility of single and multiple injections involving smaller doses or spaced over longer periods.

The present study does not provide any information about the mechanism underlying RCS-01-induced gene expression in human skin. Since RCS-01 treatment increased the expression of genes, which are primarily expressed by dermal fibroblasts, we believe that RCS-01 mainly acts at the level of the dermis. It should be noted, however, that in this study full-thickness punch biopsies were analysed for gene expression without further separation into dermal and epidermal compartments. We can therefore not rule out that RCS-01 can also have effects on epidermal cells. Increased mRNA expression was not observed in skin areas, which were injected with placebo only, or sham injected, indicating that modulation of gene expression was not due to the presence of the cryomedium or to mechanical effects caused by the injection procedure per se, but instead required the intradermal presence of the injected NBDS-derived fibroblasts. Further studies will need to clarify how these injected cells can modulate the gene expression pattern expressed in the injected skin sites.

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