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► **To cite this version:**

Melanie Velier, Alexia Mattei, Stephanie Simoncini, Jeremy Magalon, Laurent Giraudo, et al.. Paracrine Effects of Adipose-Derived Cellular Therapies in an in Vitro Fibrogenesis Model of Human Vocal Fold Scarring. *Journal of Voice*, 2022, 10.1016/j.jvoice.2022.05.012 . hal-03764296

HAL Id: hal-03764296

<https://hal.science/hal-03764296>

Submitted on 30 Aug 2022

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Paracrine Effects of Adipose-Derived Cellular Therapies in an *in Vitro* Fibrogenesis Model of Human Vocal Fold Scarring

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SUMMARY: Objectives/Hypothesis. Vocal folds (VF) scarring leads to severe dysphonia which negatively impacts daily life of patients. Current therapeutic options are limited due in large part to the high complexity of the micro-structure of the VF. Innovative therapies derived from adipose tissue such as stromal vascular fraction (SVF) or adipose derived stromal/ stem cells (ASC) are currently being evaluated in this indication and paracrine anti-fibrotic effects are considered as predominant mechanisms.

Methods. The paracrine anti-fibrotic effects of SVF and ASC from healthy donors were tested in an innovative *in vitro* fibrogenesis model employing human VF fibroblasts (hVFF) and the principles of macromolecular crowding (MMC). Biosynthesis of collagen and alpha-smooth-muscle actin (α SMA) expression in hVFF were quantified after five days of indirect coculture with ASC or SVF using silver stain, western blot and RT-qPCR analysis.

Results. Fibrogenesis was promoted by addition of transforming growth factor beta 1 (TGF β 1) combined with MMC characterized by an enhanced deposition of fibrillar collagens and the acquisition of a myofibroblast phenotype (overexpression of α SMA). Adipose-derived therapies led to a reduction in the α SMA expression and the collagen content was lower in hVFF co-cultivated with SVF.

Conclusions. ASC and SVF promoted significant prevention of fibrosis in an *in vitro* fibrogenesis model through paracrine mechanisms, supporting further development of adipose-derived cellular therapies in VF scarring.

Key Words: Vocal cords—Dysphonia—Stem cell—Fibrosis—Fibroblasts—Hepatocyte growth factor.

Abbreviations: VF, vocal fold—ECM, extracellular matrix— α SMA, alpha-smooth-muscle actin—ACTA2, actin alpha 2—bFGF, basic fibroblasts growth factor—HGF, hepatocyte growth factor—MSC, mesenchymal stromal cells—SVF, stromal vascular fraction—ASC, adipose derived stromal cells—hVFF, human vocal fold fibroblasts—MMC, macromolecular crowding—TGF- β 1, transforming growth factor beta 1—DMEM, Dulbecco's modified Eagle's medium—FCS, fetal calf serum—SM, standard medium—GMP, good manufacturing practices—RT, reverse transcription—RT-qPCR, reverse transcription quantitative polymerase chain reaction—GAPDH, glyceraldehyde-3-phosphate dehydrogenase—SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis—VEGF, vascular endothelial growth factor—COL1A1, Collagen 1 alpha 1.

INTRODUCTION

Vocal fold (VF) scar is one of the most challenging benign laryngeal pathologies,¹ especially because VF microstructure is complex,² particularly due to its foliated organization allowing optimal vibration. The proportions and the organization of its extracellular matrix (ECM) components largely determine the mechanical properties of the VF. VF scarring is most often the result of laryngeal microsurgery, but it may also be found congenitally (sulcus vocalis) or acquired

following a trauma or chronic inflammatory phenomena. Thus, the normal microstructure is replaced by fibrous tissue, with an excessive and disorganized ECM inducing vibration disorder.¹ Compared to normal fibroblasts, VF scar tissue fibroblasts produce a larger amount of collagen, proliferate more rapidly and overexpress α -smooth-muscle actin (α SMA), also known as actin alpha 2 (ACTA2), a myofibroblast differentiation marker.³

Current therapies (medialization, scar resection, microflap, injection of hyaluronic acid, etc.)^{2,4} are often disappointing since they do not restore a normal ECM distribution. Other more experimental treatments such as administration of basic Fibroblast Growth Factor (bFGF)⁵ or Hepatocyte Growth Factor (HGF)^{6,7} have shown encouraging effects *in vitro* or *in vivo* on animals. Only one phase I/II clinical trial assessed the safety and effectiveness of intracordal injection of a recombinant human HGF⁸: the results indicated no serious adverse events and encouraging clinical results. Thus, the identification of innovative strategies able to improve the mechanical vibrational properties of VF remains an important clinical challenge.

Mesenchymal stromal cells (MSC), which were first described in bone marrow to support hematopoiesis,⁹ are

Accepted for publication May 17, 2022.

This work was supported by PHC Amadeus Campus France 2017 (grant number FR05/2017).

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Journal of Voice, Vol. ■■■, No. ■■■, pp. ■■■–■■■
0892-1997

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<https://doi.org/10.1016/j.jvoice.2022.05.012>

multipotent stem cells that can be isolated from a variety of tissues but adipose tissue remains a very attractive source due to its abundance and accessibility. Autologous adipose-derived Stromal Vascular Fraction (SVF) is a heterogeneous population of cells obtained in a few hours after enzymatic digestion of adipose tissue that does not require an expansion step. SVF is also an advantageous source of adipose derived stromal cells (ASC) because of its fluidity and ease to inject. Besides, SVF brings the synergistic effect of its various cellular subpopulations, including ASC but also endothelial progenitor cells combining vasculogenic, anti-fibrotic and anti-inflammatory properties. The exact mechanism of action of ASC and SVF are not fully elucidated but it is now admitted that the major effect rests on paracrine activity through a large type of secreted molecules.^{10,11}

To facilitate research on this field, Graupp et al. described an *in vitro* fibrogenesis model using human vocal fold fibroblasts (hVFF) and the principles of macromolecular crowding (MMC).¹² MMC promotes fibrogenesis after the addition of transforming growth factor-beta 1 (TGF- β 1) to a medium containing inert macromolecules. The aim of this experimental study was to assess the *in vitro* paracrine potential of SVF cells and ASC on scarred hVFF in this innovative model.

MATERIAL AND METHODS

Donors

Adipose derived stromal cells (ASC) and Stromal Vascular Fraction (SVF) cells from healthy donors ($n = 4$) were obtained from adipose tissue surgical residues following liposuction for aesthetic purposes. All patients provided informed consent for the scientific use of surgical residues.

Cell culture

Immortalized human vocal fold fibroblasts (hVFF) were obtained from the lab of Prof. Susan Thibeault (University of Wisconsin, Madison, USA).¹³ Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Waltham, Massachusetts) supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich, Vienna, Austria) and 100 μ g/mL Normocin (Invivogen, San Diego, California, USA) was used as standard medium (SM). Adipose tissue collection was

conducted under conscious sedation with local anesthesia. SVF was obtained in a Cell Therapy Unit using the automated processing Celution 800/CRS system (Cytori Therapeutics Inc., San Diego, California, USA) as previously described.¹⁴ Collected lipoaspirate was washed and enzymatically digested to obtain SVF in accordance with the European Regulations and Good Manufacturing Practices (GMP) for 'Advanced Therapy Medicinal Products'. To isolate the ASC, SVF was then plated into a T75 cell culture flask in a culture medium consisting of DMEM supplemented with 50% Ham's F-12 Nutrient Mix (Thermo Scientific, Waltham, Massachusetts, USA), 10% FCS, GlutaMAX (100X, Thermo Scientific), Gentamicin and Penicillin G (both Panpharma, Luitré, France), Fungizone (Bristol-Meyers Squibb, New York, NY, USA). Adherent cells were grown under standard cell culture conditions (5% CO₂, 37°C, humidified), detached with Trypsin-EDTA and passed to extend the culture. For co-culture experiments, hVFF from passages 13–20 were seeded into cell culture plates (15.000 cells/cm²). The following day, SM was switched to FCS-free SM for another 24 h. Cells were then allocated to different treatment groups. For the control group DMEM enriched with 0.5% FCS, 100 μ g/mL Normocin and 100 nM of L-ascorbic acid 2-phosphate was used. Myofibroblast differentiation was induced by adding of 5 ng/mL TGF- β 1 combined with "macromolecular crowding" (MMC) by adding a mixture of 37.5 mg/mL 70 kDa Ficoll (Fc) with 25 mg/mL 400 kDa Fc (both Sigma-Aldrich). To assess possible anti-fibrotic effects, three conditions were tested in co-culture with hVFF: (i) recombinant HGF (50 ng/mL, Sigma-Aldrich), (ii) paracrine effect of thawed SVF and (iii) paracrine effect of thawed ASC from passages 3–5. After 5 days of incubation, samples were further processed.

Reverse transcription-qPCR

RNA isolation of hVFF was performed using the QIAzol Lysis Reagent (Qiagen, Hilden, Germany) and total mRNA was isolated with the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Purified RNA was eluted in RNase-free water and concentration was determined using the NanoDrop 2000c spectrophotometer (Thermo Scientific). Reverse transcription (RT), as well as

TABLE 1.
Primer sequences used for RT-qPCR

Gene	Gene symbol	Forward primer	Reverse primer	Product length [bp]
Alpha smooth Muscle action	ACTA2	CGTTACTACTGCTGAGCGTGA	GCCCATCAGGCAACTCGTAA	137
Beta-2-microglobulin	B2M	AGGCTATCCAGCGTACTCCA	CGGATGGATGAAACCCAGACA	105
Collagen I α 1	COL1A1	CCCCGAGGCTCTGAAGGT	GCAATACCAGGAGCACCATTG	140

RT quantitative PCR (RT-qPCR), was performed as previously described.¹⁵ Primer sequences are provided in Table 1. Relative quantification of all mRNAs of interest was performed based on the $2^{-\Delta\Delta C_T}$ method.¹⁶

Pepsin digestion, sodium dodecylsulphate-polyacrylamide gel electrophoresis and silver stain

Supernatants and cell layers from hVFF seeded in 24-well-plates were harvested separately and pepsinized as previously described.¹⁷ Briefly, 50 μ l of a pepsin stock solution (1 mg/mL dissolved in 1N HCL) were added to 500 μ L of supernatants, while a pepsin digestion solution (25% pepsin stock solution, 0.005% Triton X-100 in ddH₂O) was added to cell layers. All samples were incubated for 2 h followed by neutralization with 1 N NaOH.

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under non-reducing conditions using 3%–8% precast Criterion XT Tris-Acetate gels and XT Tricine running buffer (both Bio-Rad, Hercules, California, USA); electrophoresis was run for 70 min at 200 V. Vitro-Col, human collagen I solution served as collagen standard (0.16 μ g/lane). Gels were subsequently stained with the SilverQuest Silver Staining kit (Thermo Scientific) according to the manufacturer's protocol. Gel images were acquired using Quantity One software (Bio-Rad) and densitometric analysis of bands was performed using Image Lab Software, Version 5.2.

Western blot

Proteins were extracted from hVFF cell layers seeded in 6-well-plates and subjected to SDS-PAGE using 4%–12%

Criterion XT Bis-Tris Gels (Bio-Rad). SDS-PAGE was run at 200 V for 60 min, followed by electroblotting of proteins (90 min at 0.5 A and 4°C) onto Nitrocellulose membranes (Bio-Rad). Immuno-detection was carried out in Tris-buffered saline supplemented with 0.1% Tween-20 (Carl Roth, Karlsruhe, Germany) and 5% milk. Membranes were incubated overnight at 4°C with primary antibodies for detection of ACTA2 (#A5228, Sigma Aldrich, 1:1000) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; #2118C, Cell Signaling, Danvers, Massachusetts, USA, 1:5000). Subsequently, blots were incubated with secondary antibody (Goat Anti-Mouse 1:5000 for ACTA2; Goat Anti-Rabbit 1:5000 for GAPDH, both abcam, Cambridge, UK). Detection was conducted using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and the ChemiDoc Touch Imaging System (bio-rad), densitometric analysis was performed using Image Lab Software, Version 5.2.

RESULTS

Validation of the MMC/TGF- β 1 model

We first validated the myofibroblast differentiation induced by TGF- β 1 combined with MMC (MMC/TGF- β 1) through the assessment of ACTA2 and Collagen I α 1 (COL1A1) contents in hVFF. Results from RT-qPCR analysis revealed that mRNA expression of ACTA2 and COL1A1 were significantly up-regulated under MMC/TGF- β 1 condition ($P = 0.019$ and $P = 0.002$ respectively) (Figure 1A and 2A) compared to the negative condition (untreated fibroblasts). Western blot and silver stain analysis performed on hVFF protein lysates were in line with the previous data: we

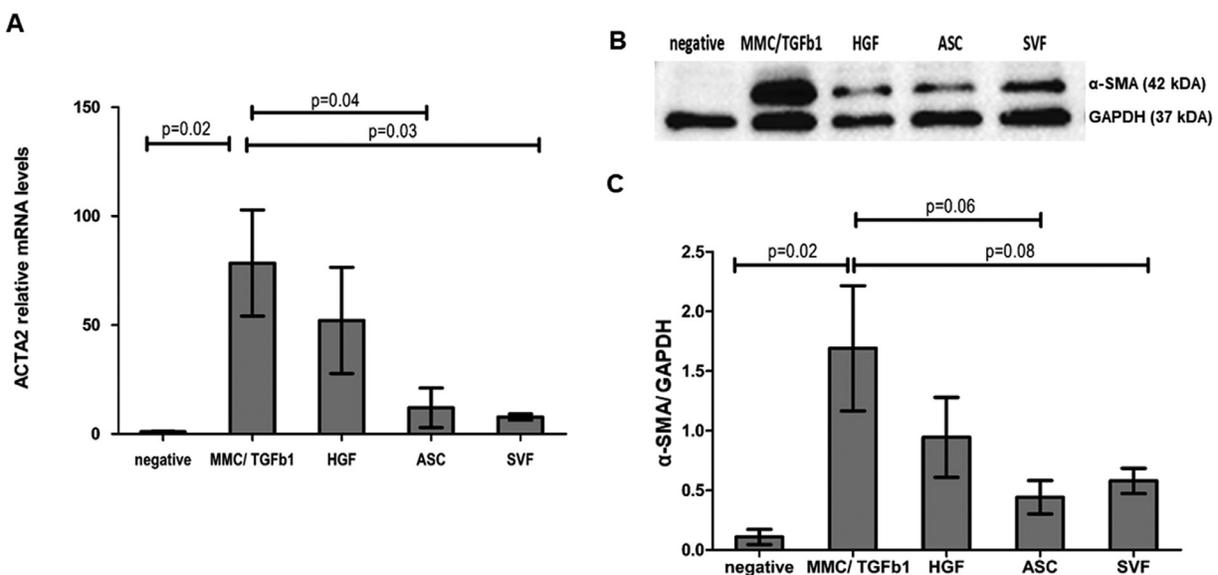


FIGURE 1. Expression of ACTA2 (also named α -SMA) (A) mRNA expression level of ACTA2 by RT-qPCR. Data is presented as mean \pm S.E.M.. Statistical analysis was performed using Mann-Whitney test. (B) The protein level of ACTA2 was examined by Western blot analysis on whole cells lysates from hVFF after co-culture with ASC (n = 4) and SVF (n = 4). Representative blots for ACTA2 and GAPDH (loading control) are shown. (C) Densitometric data from ACTA2 protein levels. Data is presented as mean \pm S.E.M., normalized to GAPDH protein levels and relative to the untreated condition (negative). Statistical analysis was performed using Mann-Whitney test.

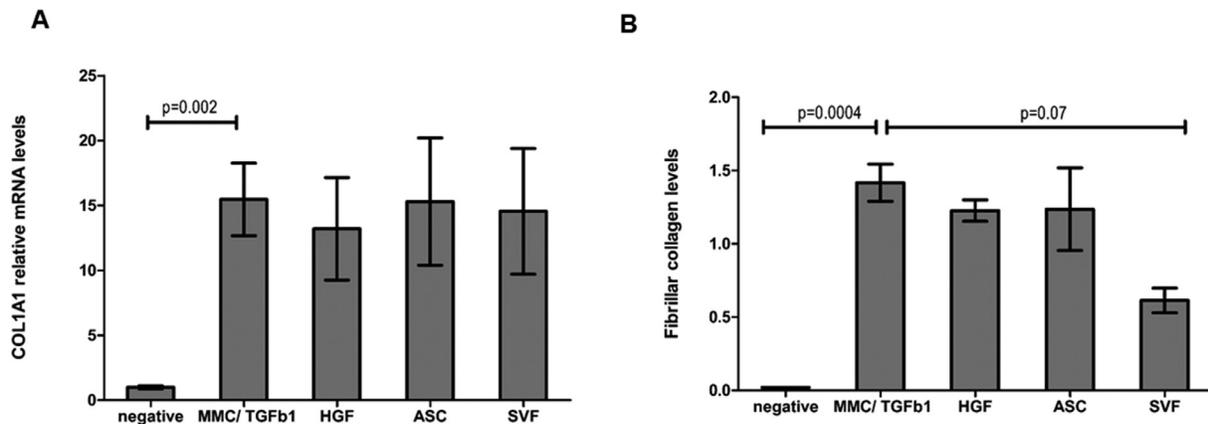


FIGURE 2. Expression of collagens (A) mRNA expression level of COL1A1 by RT-qPCR. Data is presented as mean \pm S.E.M. Statistical analysis was performed using Mann-Whitney test. (B) Densitometric data from fibrillar collagen I protein levels. Data is presented as mean \pm S.E.M., normalized to collagen I protein levels of alpha2 chain under crowded condition with TGF- β 1, and relative to the untreated condition (negative). Statistical analysis was performed using Mann-Whitney test.

observed a significant elevation of the ACTA2/GADPH ratio ($P = 0.024$) and of fibrillar collagen contents ($P = 0.0004$) in the MMC/TGF- β 1 condition compared to the untreated condition (Figure 1B and 1C). Altogether, these data validated the myofibroblast phenotype acquisition of immortalized hVFF after MMC/TGF- β 1 treatment.

Co-culture

The expression of the myofibroblast marker ACTA2 and collagen content of hVFF co-cultured with ASC or SVF or treated with HGF for 5 days were assessed. The results revealed a reduction in the mRNA level of ACTA2 on hVFF when they were co-cultured with ASC and SVF ($P = 0.043$ and $P = 0.028$ respectively) (Figure 1A). Western blot analysis confirmed these results highlighting a tendency to reduce the level of ACTA2 in hVFF co-cultured with both ASC and SVF ($P = 0.061$ and $P = 0.083$ respectively) (Figure 1B and 1C).

Figure 2 represents the expression of mRNA of COL1A1 and fibrillar collagen in protein lysates of hVFF. We observed no significant reduction in the COL1A1 mRNA expression (Figure 2A) in both ASC and SVF co-culture conditions whereas silver stain analysis revealed a significant reduction of fibrillar collagen content in hVFF after co-culture with SVF ($P = 0.006$) (Figure 2B). Of note, no significant reduction of ACTA2 or collagen levels were obtained when hVFF were treated with HGF (Figure 1 and 2).

DISCUSSION

Vocal fold scarring remains one of the most difficult conditions to treat among benign VF diseases and adipose-derived cell therapies represent hope for these patients. The therapeutic potential of MSC has largely been attributed to paracrine activity.^{18,19} In this study, we demonstrated that adipose derived stromal cells (ASC) and stromal Vascular Fraction (SVF) promoted significant prevention of fibrosis in an *in vitro* model through paracrine mechanisms.

Previously, a similar study explored whether ASC have therapeutic potential for treating vocal fold scarring through the effects of HGF on scar fibroblasts in an indirect co-culture model.³ They showed that co-culture with ASC led to a significant decrease of ACTA2 expression in fibroblasts comforting our data. However, they also demonstrated a reduction in collagen production in the ASC co-culture conditions whereas, in our study, we did not observe significant effect of ASC on the fibrillar collagen content.

Compared with ASC, SVF can be real time obtained in a sufficient quantity without *in vitro* culture, reducing drastically the delay of preparation. Yasuda et al. reported that injection of SVF had a protective effect in an acute kidney injury *in vivo* model by secreting renoprotective molecules, such as HGF and VEGF.²⁰ We can hypothesize that in the present study, SVF and ASC reduced the ACTA2 and collagen expression in hVFF partly by secreting those growth factors transferred through the co-culture system. Only conditions with SVF showed a reduction in both ACTA2 and collagen contents which might be attributed to the higher secretion of soluble mediators by SVF. In line with this, the secretome of ASC and SVF has been compared recently and revealed that SVF released a greater variety of cytokines or soluble proteins at significantly higher amounts as compared with ASC.²¹

ACTA2 was downregulated on mRNA and protein-levels, but collagen was only significantly down-regulated by SVF at the level of fibrillar collagen, that was quantified by silver stain analysis. On COL1A1 mRNA level, no reduction was detected. This could be explained by the complex extracellular assembly of different types of collagen produced by fibroblast as part of the ECM and involved in the fibrosis process.

Several *in vivo* studies on animals reported significant improvements in scarred vocal folds after injection of ASC (immediately after injury or at a longer interval) in both histological and macroscopic aspects of the vocal folds²² with an excellent tolerance of the procedure. A first clinical trial

of SVF injection which enrolled 8 patients was recently reported by our group²³ suggesting a clear improvement in the majority of the vocal parameters obtained at 12 months. However, the etiology of vocal fold scarring is very heterogeneous so a better understanding of the mechanisms underlying the efficacy of ASC and SVF is mandatory. In addition, identification of *in vitro* potency assay able to anticipate the anti-fibrotic potential of the cell therapy product could allow a better selection of patients who can benefit from these innovative therapies.

CONCLUSIONS

Based on our results, it appears that adipose derived-cellular therapies are interesting candidates for reducing myofibroblast phenotype acquisition. Nevertheless, global approaches to further characterize ASC and SVF anti-fibrotic potential and studies on a large scale are further needed to better characterize the mechanisms of action.

AUTHORS' CONTRIBUTIONS

Data interpretation: MV, AM, SS, JM, AG, FDG, FS, MG, TG; Revise the manuscript: JM, FDG, FS, MG, TG; All authors read and approved the final manuscript.

CONFLICT OF INTEREST

None.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All patients provided informed consent for the scientific use of surgical residues.

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