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Tissue Regeneration of the Vocal Fold Using Bone Marrow Mesenchymal Stem Cells and Synthetic Extracellular Matrix Injections in Rats

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Abstract

Objective—To determine the effectiveness of bone marrow mesenchymal stem cell (BM-MSC) transplantation in isolation or within a synthetic extracellular matrix (sECM) for tissue regeneration of the scarred vocal fold lamina propria.

Methods—*In vitro* stability and compatibility of mouse BM-MSC embedded in sECM was assessed by flow cytometry detection of BM-MSC marker expression and proliferation. Eighteen rats were subjected to vocal fold injury bilaterally, followed by one month post-treatment with unilateral injections of saline or sECM hydrogel (Extracel), GFP-mouse BM-MSC or BM-MSC suspended in sECM. Outcomes measured one month after treatment included procollagen-III, fibronectin, hyaluronan synthase-III (HAS3), hyaluronidase (HYAL3), smooth muscle actin (SMA) and transforming growth factor-beta 1 (TGF- β 1) mRNA expression. The persistence of GFP BM-MSC, proliferation, apoptosis and myofibroblast differentiation was assessed by immunofluorescence.

Results—BM-MSC grown *in vitro* within sECM express Sca-1, are positive for hyaluronan receptor CD44 and continue to proliferate. In the *in vivo* study, groups injected with BM-MSC had detectable GFP-labeled BM-MSC remaining, showed proliferation and low apoptotic or myofibroblast markers compared to the contralateral side. Embedded BM-MSC in sECM group exhibited increased levels of procollagen III, fibronectin and TGF- β 1. BM-MSC within sECM downregulated the expression of SMA compared to BM-MSC alone, exhibited upregulation of HYAL3 and no change in HAS3 compared to saline.

Conclusions—Treatment of vocal fold scarring with BM-MSC injected in a sECM displayed the most favorable outcomes in ECM production, hyaluronan metabolism, myofibroblast differentiation and production of TGF- β 1. Furthermore, the combined treatment had no detectable cytotoxicity and preserved local cell proliferation.

Keywords

Mesenchymal stem cells; tissue engineering; hydrogel; extracellular matrix; myofibroblast differentiation

Introduction

Vocal fold scarring is the greatest cause of voice deficiencies after vocal fold injury and can be caused by a variety of factors including, but not limited to trauma, surgical treatment, and inflammation post infection or injury 1. Current surgical and pharmaceutical interventions are often unsatisfactory and result in inconsistent short and long term outcomes 2. When a scar is formed, the normal viscoelastic properties of the vocal fold lamina propria are compromised, which leads to hoarseness, loss of vocal control, and fatigue 1. The viscoelastic properties of the vocal folds are dependent on their multilayer composition of epithelial, extracellular matrix (ECM) and muscle which are remodeled when a scar forms. The repercussions of this condition may greatly reduce an individual's quality of life by affecting fundamental work, family and social interactions. The overall goal of our study is to develop an *in vivo* tissue engineering construct which would facilitate tissue regeneration while maintaining natural biomechanical properties of the vocal fold in order to produce or reestablish unhindered phonation.

Reconstructive tissue engineering approaches employ local or allogenic cell function and diverse biomaterials as therapeutic tools to facilitate tissue healing and regeneration. Current literature in this subject suggests two general biomimetic approaches for the treatment of vocal fold scarring, biocompatible scaffolds or replacement tissue grafts 3. Scaffolds tested to promote proper healing of the vocal fold lamina propria include acellular xenogenic matrix 4,5, collagen hydrogels 6, and hyaluronic acid (HA) based hydrogels 7-10. Scaffolds should function as a space-filling material, which closely mimics the biomechanical properties of uninjured ECM components of the vocal fold lamina propria. Many authors have argued that hyaluronic acid-based hydrogels hold promise for tissue engineering applications 7-18 as they have been reported to be non-inflammatory, non-immunogenic, offer significant shock absorbing and biocompatible physical qualities. Consequently, HA-based hydrogels are potentially an ideal scaffold to improve wound healing while allowing normal cell function. One of the stumbling blocks in this research is that the biomolecular mechanism of healing through the use of scaffold injections remains to be characterized. Previous studies of prophylactic therapies have predominately reported an improvement in viscoelastic properties of injured vocal folds treated with scaffold injections when compared to saline-treated controls 8-9. However, injection of space-filling short lived materials by themselves failed to prevent the vocal fold from deforming, because of their reabsorption or remodeling over the long term 19.

The second most common method, utilizes an injection of cells to treat vocal fold scarring. Cell approaches include autologous and non-autologous mesenchymal stem cells (MSC) 20-21, autologous fibroblasts 22 and allogenic human embryonic stem cells 23. According to Liechty 24, MSC have been shown to possess site-specific differentiation, predominately into fibroblastic cells, supporting collagen synthesis, assembly and improved mechanical properties in connective tissue. Moreover, Kanemaru *et al.* demonstrated autologous MSC are easy to harvest and culture *in vitro* with low probability of rejection by the immune system 20, as they are acquired from the same patient needing treatment. Additionally, the use of MSC does not raise the same embryonic stem cell ethical concerns. The benefits of MSC therapies abovementioned make it a worthy candidate for regeneration of damaged vocal fold tissue. Both, Liechty *et al.* 24 and Kanemaru *et al.* 19 reported an improvement in healing of injured tissues injected with MSC when compared to controls however, the authors also found that the density of MSC at the site of injury was low. Consistently, it will be beneficial to improve upon strategies that can potentially enhance site-specific differentiation and healing properties of MSC. Therefore, the concurrent injection of cells and a scaffold in an injured vocal fold can favorably affect the limitations observed through the application of a single factor. In this regard, Kanemaru *et al.* studies 20 suggest the

combination of three elements: cells, scaffolds, and enhancing factors. In theory, the scaffold will initially allow the cells to be transported to and retained in the injury site; then as the scaffold is assimilated due to natural turnover processes, the implanted cells will remain at the site of the wound, resulting in newly formed tissue with similar biomechanical properties to the former tissue. We hypothesize that the interaction between BM-MSC and the hyaluronan based sECM scaffold injected simultaneously will produce a synergistic effect that enhances tissue healing and regeneration to a greater extent than either element would if injected alone.

Materials and Methods

In vitro Bone Marrow Mesenchymal Stem Cell (BM-MSC) Characterization in a Synthetic Extracellular Matrix (sECM)

The survival, proliferation and conservation of BM-MSC properties when cultured encapsulated within the sECM (Extracel, Glycosan, Salt Lake City, UT) was assessed using mouse bone marrow cells (D1 from Balb/c mice, ATCC, Manassas, VA). Cells were submerged in Dulbecco's modified eagle medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma Aldrich, St Louis, MO) and 1% antibiotic/antimycotic (Gibco, Grand Island, NY:), using 6 well plate cell culture inserts (Nalgen Nunc International, Rochester, NY), at a density of 5×10^4 cells/cm³. The sECM is a chemically modified hyaluronic acid derivate: hyaluronan, cross-linked with gelatin-DTPH (3,3'-dithiolbis(propionic hydrazide) by a poly(ethylene glycol)diacrylate (PEGDA) solution, at a ratio of 4:1, where the gelatin concentration is 5% (w/v). Detailed explanation of the procedures and hydrogel formulations have been described previously [16-18]. Cell surface expression of Sca-1, the hyaluronan receptor CD44 and viability [negative permeability to (7-amino-actinomycin D) 7-AAD] before and after 3-D *in vitro* culture were measured by flow cytometry (antibody concentrations 0.5ug/10⁵ cells). Other negative cell surface markers, indicative of purity, that were measured in these cells by flow cytometry, before and after encapsulated culture, included: CD11b, CD19 and CD-45 (primary and phyco-erithrin conjugated antibodies obtained from eBiosciences, San Diego, CA). Flow cytometry was completed on a FACS caliber (Becton Dickinson, San Jose, CA) using Cell Quest software with 10,000 events collected for each sample (Rat IgG2a isotype and all other antibodies were monoclonal, phyco-erythrin-conjugated secondary from eBioscience, San Diego, CA). Marker expression levels were initially quantified in single cell suspension. After 3 days in submerged culture the cells were harvested by gel digestion with 10 volumes of 200 U of hyaluronidase/collagenase-I (45 minutes at 37°C while shaking). Then, DMEM 10% FBS was added to stop the enzymatic process and the resulted suspension was filtered through a mesh (70 µm) to separate released cells from undigested gel fragments. Harvested cells were washed by centrifugation with PBS containing 1% FBS solution to remove enzyme and/or hydrogel remnants.

Alamar Blue assay was used to quantitatively measure the number of viable cells due to proliferation, 24, 48 and 72 hours after initial seeding in isolation and in sECM. Briefly, 10% v/v of Alamar Blue was added to the culture media and incubated for 4 hours. Subsequently, 100 µL of media were taken from each culture and tested in a 96 well plate for their fluorescence levels (proportional to the number of viable cells) using a Multi-Detection Microplate Reader (Bio/Tek FLx800). Fluorescence was measured at an excitation wavelength of 530 nm and emission wavelength of 590 nm. Microplate Data Analysis Software (KC 4 from Bio-Tek. Winooski, VT) was used for quantitative analysis. Triplicate samples of a blank control (plain culture medium with added Alamar Blue) were run simultaneously. Direct exposure to light and heat was circumvented during these procedures.

In vivo tissue regeneration in scarred rat vocal folds

Tissue regeneration in an injured rat vocal fold scar model was assessed for 4 treatment groups: injection with mouse enhanced green fluorescent protein (eGFP) BM-MSC, injection with Extracel as a sECM, injection with eGFP BM-MSC embedded in sECM and injection of saline. Outcome measures included transcription analysis of the ECM proteins, hyaluronan metabolizing enzymes, and wound healing/remodeling factors. Furthermore, we determined the presence of remaining GFP grafted cells, proliferation, apoptosis and smooth muscle actin expression in the lamina propria by immunofluorescence.

Mouse eGFP BM-MSC were obtained by flushing the contents of both femurs from nine eGFP expressing Balb/c mice (weighting 20- 25 g), using DMEM, onto a 10 cm tissue culture plate followed by mechanical disaggregation by multiple aspirations through a 27 gauge needle. The mice were pre-medicated with ketamine and xylazine (according to their weigh) and then sacrificed for the BM extraction procedure. Subsequently, the BM-MSC were grown as described above for the D1 BM MSC cell line on T-25 flasks, and extended up to 4 passages. All animal experiments used in this study were performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH *Guide for the Care and Use of Laboratory Animals*, and the Animal Welfare Act (7 U.S.C. et seq.). The animal use protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Wisconsin-Madison.

Vocal Fold Biopsy

Twelve Rowett nude rats were anesthetized by exposure to isoflurane (3% delivered at 0.8–1.5 L/minute) and intra-peritoneal injection of 90 mg/Kg of ketamine hydrochloride, 9 mg/Kg of xylazine hydrochloride. Intra-peritoneal atropine was used to reduce salivation (40 ug/Kg). Microscopic direct laryngoscopy was performed using a custom made steel wire oral stabilizer to maintain laryngeal access²⁵, an endoscope (1.9-mm diameter 30° endoscope from KARL STORZ Endovision, Inc., Charlton, MA; connected to an external light source and video monitor) and an operating microscope. In all rats, scars were created bilaterally with a 27-gauge syringe needle on the mid-membranous point of the vocal folds. Postoperative analgesia was administered for 24 hours using 0.01 mg/Kg of buprenorphine. One month after biopsy, rats were anesthetized as described above and the right vocal fold scars were injected with BM-MSC alone, BM-MSC embedded in sECM, or sECM alone (4 rats in each group) and saline on the contra lateral side. The rats were anesthetized and sacrificed (Euthazol 0.05 mg/Kg intra-cardiac injection) one month after treatment and the larynges were excised for evaluation of histology, immunohistochemistry and gene expression after cryo-section or dissection, respectively.

Gene Transcription

Similar methodology for gene transcription analysis is described elsewhere^{8,9}. In order to analyze the mRNA, the tissue was homogenized and total RNA was extracted using the UltraClean™ Tissue RNA Isolation Kit according to the manufacturer's instructions (ISCBiosciences/Intermountain Scientific Co., Salt Lake City, UT). Total RNA concentration was measured by spectrophotometric analysis using a nanodrop 1000 (Thermo Scientific, Fremont, CA), and the quality of the RNA was assessed through gel electrophoresis. With extracted total RNA, real time PCR was carried out as previously described²⁶, using LightCycler® FastStart DNA MasterPLUS SYBR Green I (Roche Diagnostics Corporation, Indianapolis, IN) in order to quantify mRNA expression of the following genes: pro-collagen III (C-III), fibronectin (FN), hyaluronan synthase (HAS3), hyaluronidase (HYAL3), smooth muscle actin (SMA), and transforming growth factor- beta 1 (TGF-β1). The primers used are described in Table I. Briefly, total RNA was isolated from the biopsies, and treated with DNase I solution, followed by inactivation of the DNase I.

Copy DNA (cDNA) was then prepared by reverse transcription (Invitrogen Superscript II). Real-time RT-PCR was performed in 20 μ L reactions containing 50 ng of cDNA as template, using a LightCycler 1.5 (Roche Diagnostics Corporation, Indianapolis, IN). After amplification, a melting curve was acquired to confirm PCR product specificity. For each gene, an 8 point 10-base dilution standard curve was constructed from purified PCR products to determine reaction efficiency and subsequent quantification of mRNA from the biopsy samples. Negative controls with water instead of template and initial mRNA were included in each run to rule out DNA and genomic DNA contamination. The LightCycler software (version 4.0) was used to calculate the concentration for the unknown samples. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified for each cDNA sample for normalization of the results obtained for each other target gene. The data obtained from absolute quantifications was used to compare the differences between the vocal folds from each treatment group.

Histology/Immunofluorescence

Immunofluorescence detection of remaining grafted eGFP BM-MSC was performed by staining with anti-GFP primary rabbit polyclonal antibody (1:400 dilution, Abcam ab290, Cambridge, MA) or rabbit IgG from the same source, followed by Alexa Fluor 594-labeled goat anti-rabbit (1:250 dilution, Invitrogen A-11012, Carlsbad, CA). Mouse eGFP staining allowed us to discriminate between BM-MSC cells that were transplanted into the vocal folds versus those MSC that have migrated in response to the injury or treatment. Proliferation was tested by staining with rabbit anti Ki-67 (1:100 dilution, Thermo Scientific clone Sp6, 9106-s, Fremont, CA) followed by TRITC-labeled goat anti rabbit secondary (Invitrogen, Carlsbad, CA). Apoptotic cells were fluorescently stained using FITC labeled dUTP and dUTP-transferase to detect DNA nicks (#11684795910 Roche applied Biosciences, Mannheim, Germany). Myofibroblasts were detected using rabbit anti SMA (1:400 dilution, Thermo Scientific, Fremont, CA) followed by TRITC labeled goat anti-rabbit secondary (1:250 dilution, Invitrogen, Carlsbad, CA). The immunofluorescence stains were controlled with the corresponding isotype control and the total number of cells within the lamina propria was evaluated by addition of 4',6-diamidino-2-phenylindole (DAPI nuclear stain, Invitrogen, Carlsbad, CA) to the secondary antibody at a dilution of 1:10,000 or to all the sections with apoptotic staining. Positive apoptotic staining was confirmed by DNase I (Qiagen, Valencia, CA) digestion of the sections (10U/30 min/37°C) and assessment of background fluorescence was performed by incubation with FITC labeled dUTP alone. Stained slides were mounted using ProLong Gold (Invitrogen, Carlsbad, CA) and a clear resin sealant to preserve fluorescence. Additionally, hematoxylin/eosin staining was used for anatomical orientation of the extent and morphology of the rat lamina propria as a reference for immunofluorescence studies (Fisher Scientific, WI, USA).

Microscopy

Inverted fluorescence microscopy was used to capture images using a Nikon Eclipse E600 microscope (Nikon, Melville, NY) and a Pixera color camera (Pixera, Los Gatos, CA), maintaining equal exposure times and post image processing for both left and right vocal fold sections, as well as stained and isotype controls, using the DP2-BSW software package (Olympus, Center Valley, PA). The number of GFP, SMA, Ki-67 or apoptotic (Tunel) stained cells and areas will be quantified above background using the Metamorph Image Analysis Software (Universal Imaging, West Chester, PA) for control and treated vocal folds. The lamina propria was examined, beginning with the superficial layer under the epithelium, and progressing to the border of the vocalis muscle. Each sample was normalized to either the total area or the total number of cells detected to allow comparison between different samples, regardless of size of the lamina propria. Intra-rater reliability was

estimated by blinded second quantification of the SMA immunofluorescence stain ($\kappa > 80\%$).

Statistical Analysis

For the purpose of immunofluorescence quantifications, the number of positive cells for each stain was determined using the same range in intensity measurements above isotype control (background) across all samples within the same stain, using the Metamorph Software Package Cell count tool (Leeds Precision Instruments, Minneapolis, MN). The obtained number of positive cells or the areas of positive staining were then normalized to the total cells present within the lamina propria as determined by positive nuclei staining (DAPI, blue) or the percentage of total lamina propria area (μm^2) (measured with the DP2 Olympus Software, Leeds Precision Instruments, Minneapolis, MN) plus or minus standard deviation of 3 separate sections from each rat (obtained in Microsoft Excel) and differences between treatment and controls were analyzed using a paired, 1-sided t-Test. The mRNA gene expression data was evaluated using the ANOVA with the Fisher's Protected Least Significance Difference (LSD) in the SAS/STAT software package. A p value of <0.05 was used to designate statistical significance.

Results

Mouse BM-MSC cultured in sECM preserve the expression of extracellular markers, retains viability and mitotic activity

Comparison of flow cytometric studies of cell surface markers before and after growing mouse BM-MSC in the hyaluronan-based sECM for 3 days were comparable. After encapsulation we observed high viability (96% or better), the newly discovered expression of CD-44 (Hyaluronan receptor, as high as 92%), as well as, a 60% retention of the cell surface marker Sca-1 (Figure 1). Additionally the BM-MSC preserved their cell growth as measured over a 3 day period with the non-cytotoxic fluorescent label Alamar blue (Figure 2B). Expression of other hematopoietic markers (CD11b, CD-19 and CD-45), remained negative before and after culture (data not shown).

Combined injection of BM-MSC and sECM produced the most favorable extracellular matrix gene expression profile

After mRNA extraction and reverse transcription the resulting cDNA was amplified using specific primers to detect C-III and FN expression and normalized to the GAPDH house-keeping gene (amplification efficiencies within 5%). As a result (Figure 2), the combined treatment with BM-MSC and sECM displayed higher levels of C-III expression than saline ($p < 0.0053$), or either sECM or BM-MSC alone ($p < 0.001$). Additionally, individual treatment with either BM-MSC or sECM were also lower in C-III expression than saline vehicle ($p < 0.001$) and were not significantly different from each other ($p < 0.9809$), as shown in Figure 2 A. Regarding FN mRNA expression, a similar trend was found, with the highest expression being detected in the rats with combined BM-MSC and sECM treatment compared to saline ($p < 0.0001$) and to individual treatments. The lower production of FN was detected in the vocal folds injected with sECM alone compared to saline ($p < 0.0001$) and all other groups. Interestingly, BM-MSC injection was not different in the stimulation of FN mRNA than saline treatment ($p < 0.0965$). All together these results indicate that injection of BM-MSC with a sECM upregulated CIII and FN mRNA expression compared to controls or individual effects.

Combined injection of BM-MSC and sECM produced a synergistic effect in hyaluronan metabolizing enzymes gene expression compared to individual treatment

HAS3 and HYAL3 mRNA expression was increased by combination of BM-MSC and sECM compared to each treatment alone ($p<0.0099$ and $p<0.0001$ respectively), as displayed in Figure 2 B. Expression of HAS3 in the combined injection was comparable to saline treatment ($p<0.1827$) indicating that injection with BM-MSC embedded in sECM did not adversely affect the synthesis of hyaluronans. In the case of HYAL3, combination treatment increased hyaluronan degradation as measured by the increase in HYAL3 mRNA expression, compared to saline treatment ($p<0.0025$), BM-MSC ($p<0.0005$) or sECM alone ($p<0.0001$). There was no significant difference between BM-MSC alone and saline treatment ($p<0.2574$) for this enzyme. These results indicate that combined treatment induces the metabolism of hyaluronan however, there is a higher effect on hyaluronan degradation than synthesis compared to vehicle injections.

Combination of BM-MSC with sECM did not increase myofibroblast differentiation while favoring TGF- β 1 release

The highest myofibroblast marker expression (alpha smooth muscle actin mRNA-SMA-) was observed when BM-MSC were injected alone compared to saline ($p<0.0001$) or combined BM-MSC plus sECM ($p<0.0001$), as shown in Figure 2C. Interestingly, sECM alone was not different from saline ($p<0.2506$) or combination with BM-MSCs ($p<0.0117$). Regarding expression of the wound healing factor TGF- β 1, figure 2C indicates that there was an increase in mRNA expression with combined BM-MSC and sECM treatment compared to saline ($p<0.0001$) or each factor alone ($p<0.0001$ in both cases). Injections with BM-MSC alone also induced higher TGF- β 1 mRNA expression than saline ($p<0.0003$); sECM showed lower expression than saline ($p<0.002$), indicating that the synthetic hydrogel also has a synergistic effect on BM-MSC-induced TGF- β 1 mRNA expression.

Immunofluorescence analysis of the lamina propria revealed persistence of GFP expression, cell proliferation, low cytotoxicity and low myofibroblast differentiation of combined BM-MSC/sECM therapy

Mouse GFP-expressing cells were detected in the athymic rats at the time of sacrifice (30 days after treatment), as shown in figure 3. Persistence of GFP grafted cells was more evident ($p=0.06$) in combination therapy (mean: 53 % area $\pm$ 28% SD) compared to BM-MSC injections alone (mean: 4.5% area $\pm$ 3% SD). Cell proliferation was detected with staining of the nuclear factor Ki-67. As displayed in figure 4, BM-MSC showed a trend for higher proliferation than when injected in conjunction with sECM (mean normalized positive cells 70 $\pm$ 9.3 SD vs 5 $\pm$ 0.8 SD, respectively). Although, there was no significant difference between treated BM-MSC/ $\pm$ sECM compared to saline controls ($p=0.23$ and $p=0.21$). Similarly, there was no detectable increase in apoptosis staining compared to saline controls (BM-MSC mean: 8 vs saline mean: 15 $\pm$ 5 SD, $p=0.13$; BM-MSC+sECM mean: 55 vs. saline mean: 54 $\pm$ 5.9 SD, $p=0.31$) with either treatment or combination (Figure 5). Regarding myofibroblast differentiation, figure 6 indicates that there was no significant increase in myofibroblast differentiation compared to saline controls (BM-MSC $p=0.2$, BM-MSC+sECM $p=0.5$) as measured by the number of SMA expression positive cells.

Discussion

The lack of long term studies and few currently used therapies for vocal fold scarring that return normal phonation and vocal fold viscoelastic properties has led us to investigate and test new materials and more integral therapeutic approaches to provide ECM and cellular stability for adequate function. The results of this study show that mouse BM-MSC are compatible with the sECM, that not only serves as a delivery system, but also is moldable to

replace the physiological anatomic space lost, and it is sufficient to sustain the grafted cells *in vitro* and *in vivo* (as GFP cells can be tracked 30 days post treatment). Furthermore, we found an enhancing effect of the combination therapy (BM-MSC plus sECM) regarding ECM production and metabolism; as well as a downregulation in myofibroblast differentiation compared to BM-MSC alone. The combination therapy also showed a higher effect on TGF- β 1 mRNA production. The effects of TGF- β 1, although controversial, are well recognized as a secondary stimuli and modulatory factor of wound healing (by increasing collagen synthesis and cell proliferation in fibroblasts and epithelial cells). In terms of myofibroblast differentiation, TGF- β has been proposed as a signal for SMA expression in connective tissue fibroblasts. However in this study, perhaps the amount of TGF- β 1 mRNA produced is post-translationally regulated (at the levels of protein expression, degradation and secretion) and/or it does not reach the threshold necessary to induce significant myofibroblast differentiation to be detectable by immunofluorescence *in vivo*.

The factors regulating remodeling versus wound healing/regeneration, are naturally produced by the body in response to an injury and include extracellular matrix proteins and enzymes such as: pro-collagen III, fibronectin, HAS3, HYAL3; and synthesis of myofibroblast differentiation proteins and factors such as SMA, and TGF- β 1. The results of gene expression in this current study are consistent with reports from previous studies 8-27; both of which indicate increased levels of gene expression for pro-collagen III, fibronectin, and TGF- β 1 during wound repair as favorable responses expected from a good therapeutic and functional outcome with the limitations that there are few quantitative correlation studies between the expression of these proteins and functional parameters. For instance, increased levels of TGF- β 1 have been found concomitantly with increased HA synthesis in vocal folds and a particular study, Chan *et al.*, 14 illustrated that in the presence of HA, vocal folds displayed improved elasticity and viscosity, which corresponds to advantageous biomechanical properties. Additionally, Duflo *et al.* 8 proposed that increased expression of procollagen III and fibronectin might relate to improved vocal fold function during the acute healing process. Currently, however, the precise role of each of these constituents in wound healing remains unknown and requires further investigation. In the present studies, gene expression profiles were favorable in the BM-MSC plus sECM for the production and metabolism of ECM proteins and the histological studies revealed little to no effect on remodeling as detected by myofibroblast differentiation or cytotoxicity (i.e. normal proliferation and no significant increase in apoptosis staining).

It is not surprising that treatment with BM-MSC alone displays greater SMA gene expression than the combination therapy or all other groups, given the ability of BM-MSC to differentiate into multiple cell types, including smooth muscle 19-24. However, when present in excess, smooth muscle may compromise the optimal biomechanical properties of the vocal fold. Accordingly, high levels of SMA gene expression present a notable disadvantage for BM-MSC only injections. Interestingly, co-administration of Extracel, our sECM, with BM-MSC dramatically reduces gene expression levels of SMA, making co-treatment with this sECM an appealing option.

Physical constraints imposed on this study inhibited the acquisition of data from longer and shorter time points, however, our immunohistochemical analysis displays the presence of implanted BM-MSC in vocal folds 30 days after injection. This demonstrates that implanted BM-MSC may have largely differentiated into vocal fold tissue types *in vivo* and some remain viable at least a month post-treatment. Visual inspection and evaluation of the H & E stained vocal folds suggests improved cellularity and maintenance of the normal tissue architecture within vocal folds injected with BM-MSC and sECM compared to saline controls (data not shown). Further studies will address a more comprehensive morphological

and quantitative evaluation of the vocal fold lamina propria based on these encouraging results. We expect to find an increase in the morphological stability of treated vocal folds, as well as an steady improvement in functional parameters, at longer time points compared to control treated tissues.

Conclusions

Injections of injured vocal folds from rats with BM-MSc appears to be beneficial in the context of a sECM, in promoting ECM deposition and growth factors production without increasing myofibroblast differentiation and therefore preserving the qualities and biological competence of the replaced tissue. Thus, grafting of BM-MSc in injured vocal folds embedded in a vehicle of hyaluronan based reabsorbable sECM represents an attractive therapeutic strategy for future clinical trials.

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Figure 1. Characterization of mouse bone marrow (D1) BM-MSC in a sECM three-dimensional culture

Flow cytometry histograms of A: Negative uptake of the cell dead dye, 7-AAD; B: Proliferation of mouse BM-MSC in sECM by Alamar Blue, C: Positive expression of the hyaluronan receptor CD-44; and D: Positive expression of the mouse BM-MSC marker, Sca-1. In these the histograms, the x axis represents cell count and the y axis represents fluorescence in a 4 decade scale. Percentage of cell staining is presented on each histogram.

Figure 2. Comparison of total mRNA gene expression in the lamina propria, as measured by quantitative real time PCR analysis in saline (S), BM-MSD (M), sECM (E) and BM-MSD+sECM (M+E) treated vocal folds

A: ECM proteins, B: Hyaluronic acid metabolic enzymes HAS3 and HYAL3, C: Wound healing and myofibroblast differentiation protein markers (TGF-β1, SMA).

Figure 3. Mouse bone marrow GFP grafted cells (red) persist 30 days post-injection as determined by indirect immunofluorescence using Alexa-labeled anti-GFP and nuclei staining (blue)
Immunofluorescence pictures of the lamina propria of BM-MSC+sECM and BM-MSC injected rats (20×)

Figure 4. In vivo assessment of cell proliferation by fluorescence staining of the mitotic marker Ki-67 (red)
Saline and treated: BM-MSC+sECM (top), and BM-MSC alone (bottom).

Figure 5. In vivo biocompatibility of sECM and/or BM-MSC injections
 (20× pictures of sECM+BM-MSC and BM-MSC). TUNEL fluorescence staining (green):
 Saline and BM-MSC/sECM-treated (top); saline and BM-MSC alone (bottom). Negative
 (FITC-dUTP) and positive (DNase-treated) controls are included.

Figure 6. In vivo evaluation of myofibroblast differentiation by SMA (red) expression after treatment
Saline and treated: BM-MSCs+sECM (top), and BM-MSCs alone (bottom).

TABLE I

Q-PCR targets and primer sequences for the rat genome.

TARGET	LEADING	LAGGING
TGF- β 1	5'-tttgagcctggacacacagta-3'	5'-tgttggacaactgctccacctt-3
SMA	5'-aatattctgtctggatcgcggtct-3'	5'-agcatttgcggtggacaatgga-3'
FIBRONECTIN	5'-atgatgaggtgcacgtgtgt-3'	5'-gatggggtcacattccatc-3'
PROCOLLAGEN-III	5'-ccattgctggagttggaggtgaaa-3'	5'-agggtggcagaatttcaggtctct-3'
HAS-3	5'-tgagtcagtggtcacaggcttct-3'	5'-agatcatctctgcattgcctcgga-3'
HYAL-3	5'-aacagctccacaagcccaactg-3'	5'-gccatattatgccagccattgcca-3'