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RECONSTRUCTION OF VOCAL FOLD SCARRING WITH MESENCHYMAL STROMAL CELL THERAPY

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RECONSTRUCTION OF VOCAL FOLD SCARRING WITH MESENCHYMAL STROMAL CELL THERAPY

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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కర్మణ్యేవాధికారస్తే మా ఫలేషు కదాచన |
మా కర్మఫలహేతుర్భూర్మా తే సఙ్గోఽస్త్వకర్మణి ||

Karmanye Vadhikarste Ma Phaleshu Kadachana
Ma Karmaphala HeturBhurma Te Sagjgosstvaa Karmani

You have the right to do your actions, but you should not worry about the results (*Bhagavad Gita, Chapter 2-Stanza 47*)

Dedicated to the people who have lost their voice or suffer with voice problems

ABSTRACT

Tissue injury/scarring of the vocal folds (VFs) can be caused by various factors including external trauma, cancer treatment or inflammation, leading to damage within the vibrating layers and a decrease in viscoelastic functions. These effects ultimately result in severe voice problems. This condition leads to significant morbidity for patients. Approximately 5-20% of the population in Western countries suffer from chronic hoarseness and/or a voice disorder. Currently, there are no effective treatments available.

Embryonic stem cells (ESCs) are pluripotent cells that can different into various cell types or tissues in the body. They are powerful candidates for cell therapy for many diseases. Mesenchymal stromal cells (MSCs) have been considered as a valuable source in regenerative medicine due to their immunomodulatory properties, ability to self-renew and multipotency. The overall aim of this PhD project was to identify whether stem cells, and in particular local injection of MSCs can modulate the early inflammatory response within scarred VFs, and promote the wound healing process. We have used a combination of *in vitro* and *in vivo* models to address the fundamental questions, before translating our findings to a clinical phase I/II trial evaluating the safety and efficacy of autologous MSC injection on vocal function in patients suffering with severe voice problems.

In **study I**, in a xenograft model, we showed that local injection of human embryonic stem cells (hESCs) induces sustained enhanced healing properties in injured VFs. Measurement of tissue viscoelasticity demonstrated improved function of VFs. Histological findings show a reduction in type I collagen and lamina propria (LP) thickness in hESC treated VFs compared to untreated VFs. Moreover, no hESCs derivates (teratoma and malignances) were observed in the transplanted VFs.

In **study II**, we examined how MSCs modulate the early inflammatory response within injured rabbit VFs. Histological examination demonstrated that injected MSCs were able to reduce tissue inflammation, further corroborated by findings confirming a significant reduction in mRNA expression of the pro-inflammatory markers, interleukin (IL)-1β and IL-8. These findings were attributed to the ability of MSCs to reduce the level of cell death at the injury site and promote an anti-inflammatory milieu, as evidenced by increased levels of CD163+ cells. Despite low level persistence of the MSCs within the tissue, long-term effects on tissue repair were evident, with improved organization of the collagen matrix.

Limitations, in terms of cell suspension leakage and retention of MSCs at the site of injury have been reported with the use of liquid delivery vehicles. **Study III** therefore addressed these issues, evaluating the effectiveness of MSC delivery to injured VFs in a hyaluronan (HA) hydrogel carrier. Our findings demonstrated that HA was non-immunogenic, with no adverse events reported, and degraded within the VF tissue within 1 month. Complementary *in vitro* studies demonstrated that while the HA hydrogel delayed the migration of MSCs, these cells remained responsive to their extracellular environment, including pro-inflammatory cues. The presence of a collagen matrix, as seen within the VF tissue, enhanced movement out of the gel potentially explaining why hydrogel delivery did not enhance persistence of the MSCs in the tissue. The study concluded that HA hydrogels may offer a safe and practical means to deliver MSCs to the VF, with minimal leakage, but no additional therapeutic effect could be seen in comparison to MSCs in liquid suspension.

Based on the findings from studies II and III, we performed a phase I/II clinical trial in 16 patients with severe VF scarring suffering from severe voice problems (**study IV**). Patients

were treated with a single injection of autologous MSCs, after surgical resection of scar tissue. The study reached its primary endpoint of safety, with no adverse side effects reported. Clinical evaluation using high speed laryngoscopy and phonation pressure threshold demonstrated improvement in VF viscoelastic function in 62-75% of patients, dependent on the parameter. Self-reporting by the patients, using the voice handicap index confirmed significant improvements in phonation in 50% of the patients, with no significant change in the remaining cohort.

In summary, stem cells and stromal cells were found to improve healing in scarred VFs. Local administration of MSCs into injured/scarred VFs dampens inflammatory responses by decreasing cell death due to injury and promoting an anti-inflammatory milieu. Delivery of the MSCs in a HA hydrogel reduces cell leakage, but does not offer therapeutic advantages compared to MSCs alone. Despite low-level persistence and no engraftment within the VF tissue, MSCs exerted long-term effects resulting in functional tissue repair. Our *in vivo* animal studies demonstrating the mechanisms by which MSCs support tissue repair were translated to the clinical setting, with local administration of autologous MSCs improving VF function and reducing morbidities associated with poor phonation in the treated patients. We conclude that local injection of MSCs may offer a safe and novel option for the treatment of VF scarring.

LIST OF SCIENTIFIC PAPERS

I. Svenssion B, **Nagubothu SR**, Nord C, Cedervall J, Hultman I, Ährlund-Richter L, Tolf A, Hertegård S.

Stem cell therapy in injured vocal folds: A three-month xenograft analysis of human embryonic stem cells.
Biomed Research International, 2015:754876.

II. **Nagubothu SR**, Sugars RV, Tudzarovski N, Andrén AT, Bottai M, Davies LC, Hertegård S, Le Blanc K.

Mesenchymal stromal cells modulate tissue repair responses within the injured vocal fold.
Laryngoscope 2019 Mar 5. doi: 10.1002/lary.27885

III. Hertegård S, **Nagubothu SR,** Malmström E, Ström CE, Tolf A, Davies LC*, Le Blanc K*.

Hyaluronan hydrogels for the local delivery of mesenchymal stromal cells to the injured vocal fold.
Stem Cells and Development. 2019 Sep 1;28(17):1177-1190
** Joint senior author*

IV. Hertegård S, **Nagubothu SR**, Malmström E, Le Blanc K.

Treatment of vocal fold scarring with autologous bone marrow derived human mesenchymal stromal cells- first phase I/II human clinical study.
Manuscript

ADDITIONAL CONTRIBUTIONS

Hertegård S, Larsson H, **Nagubothu SR**, Tolf A, Svensson B. Elasticity measurements in scarred rabbit vocal folds using air pulse stimulation. *Logopedics Phonatrics Vocology, 2009 May 1:1-6*

Svensson B, **Nagubothu SR**, Cedervall J, Le Blanc K, Ährlund-Richter L, Tolf A, Hertegård S. Injection of human mesenchymal stem cells improves healing of scarred vocal folds: analysis using a xenograft model. *Laryngoscope, 2010 Jul; 120(7): 1370-5*

Svensson B, **Nagubothu SR**, Cedervall J, Chan RW, Le Blanc K, Kimura M, Ährlund-Richter L, Tolf A, Hertegård S. Injection of human mesenchymal stem cells improves healing of vocal folds after scar excision--a xenograft analysis.
Laryngoscope, 2011 Oct; 121(10): 2185-90.

CONTENTS

LIST OF ABBREVIATIONS

ADSCs	Adipose-derived stromal cells
αSMA	Alpha smooth muscle actin
bFGF	Basic fibroblast growth factor
BM-MSCs	Bone marrow derived mesenchymal stromal cells
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
DAB	3,3'-Diaminobenzidine
DAPI	4′,6-diamidino-2-phenylindole
DLP	Deep lamina propria
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
ESC	Embryonic stem cell
FCS	Fetal calf serum
FISH	Fluorescence *in situ* hybridization
GAG	Glycosaminoglycan
H & E	Hematoxylin and eosin
HA	Hyaluronan
HAS	Hyaluronan synthase
HBV	Hepatitis B virus
HCV	Hepatitis C virus
hESC	Human embryonic stem cell
HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HSS	High-Speed Studio software
HTLV	Human T lymphotrophic virus
HYAL	Hyaluronidase
ICM	Inner cell mass

IDO	Indoleamine 2,3-dioxygenase
IF	Immunofluorescence
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
iNOS	Inducible nitric oxide synthase
ISCT	International Society of Cell and Gene Therapy
LP	Lamina propria
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage colony stimulating factor
MLP	Middle layer of lamina propria
MMP	Matrix metalloproteinase
MSC	Mesenchymal stromal cell
P	Passage
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PGE2	Prostaglandin E2
PTP	Phonation pressure threshold
PVA	Poly-vinyl alcohol
qPCR	Quantitative polymerase chain reaction
SDF-1	Stromal cell-derived factor-1
SLP	Superficial lamina propria
SRCR	Scavenger receptor cysteine-rich
TGFβ1	Transforming growth factor beta 1
VF	Vocal fold
VEGF	Vascular endothelial growth factor
TLR	Toll like receptor
TNF-α	Tumor necrosis factor alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

VHI	Voice handicap index

1 INTRODUCTION

Vibration of the vocal fold (VF) tissue within the larynx is important for voice production, and dependent on neuromuscular activity for gross movement and pliability during phonation(1). VF scarring, due to external trauma, radiation, iatrogenic causes, inflammation or surgical treatment for neoplasms, is considered to be the most common cause of severe voice problems(2). Abnormal wound healing within VF tissue results in scar formation due to large-scale replacement of the superficial layer of the extracellular matrix (ECM) with collagen type I deposition in the lamina propria (LP), interrupting the delicate balance between the viscosity of the superficial layer of the VFs and increasing tissue stiffness(3). The voice, in turn, becomes aphonic or breathy and the phonation threshold pressure (PTP), corresponding to ease of phonation, is elevated(4). Along with viscoelastic changes in the scar tissue, the shear strength of the VFs also changes so that the normal mucosal wave is diminished, or even absent, during phonation. This results in a loss of voice control, hoarseness and vocal fatigue(5).

1.1 ANATOMY OF THE VOCAL FOLD

The microstructure of VF tissue is complex due to its foliated organization. According to the body-cover theory proposed by Hirano(6), the VFs consist of a superficial layer (cover) comprised of epithelium, basal membrane and superficial lamina propria (SLP). The inferior layer (body) consists of deep lamina propria (DLP) connected to thyroarytenoid muscle, separated by a middle layer of lamina propria (MLP; Figure 1). This distinct architecture enables the two functional units to vibrate individually, with the level of vibration influenced by many factors such as loudness, pitch and age(7).

The junction between the epithelial layer and LP is known as the basement membrane zone or lamina densa, and consists of structural proteins such as type IV collagen. The SLP is found below the basement membrane zone, a region called Reinke's space, which plays a crucial role in separating the mucosal cover from the VF body(8). The SLP layer contains loose connective tissue with few type III collagen or elastin fibers, whereas the MLP and DLP layers are characterized by a higher amount of elastin and collagen respectively(7).

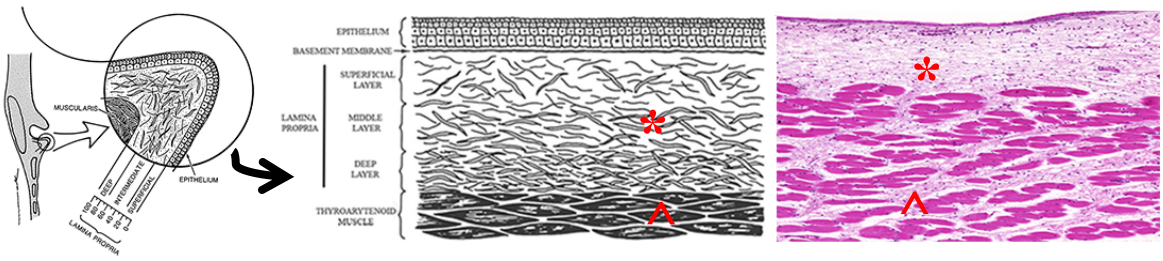

Figure 1: Schematic and histological images illustrating the layers of the vocal fold tissue. (A and B) Schematic illustration highlighting the layered structure of the vocal fold tissue. (B) Histological section through the rabbit vocal fold tissue, highlighting the epithelial, lamina propria (*) and thyroarytenoid muscle (^) layers. The deeper muscular layer is noted in the rabbit compared to human vocal fold tissue. Figure adapted with permission from *Gray et al., 2000, Ann Otol Rhinol Laryngol 109: 77-85(7)*.

This layered structure is found in the freely vibrating membranous mid-part of the VF tissue, but the structure changes close to the attachment of the VF tissue in the anterior and posterior regions where the macula flavae is located. The macula flavae or nodulus elasticus is comprised of dense cellular masses, which together with their ECM, are necessary for conveying the viscoelastic properties of the VF. ECM components such as reticular, elastic collagenous fibers(9), and hyaluronan (HA)(10) are important for the vibratory functions of the VF. Stellate cells resident in the macula flavae show morphological and phenotypic differences from stromal fibroblasts in the VFs(11, 12). These cells have well developed intracellular organelles, and when activated, continuously synthesize LP ECM, and play an important role in both acute and chronic inflammation(13-15).

Rabbit VFs are relatively similar to human VFs, based on their ultrastructure, ECM content and viscoelastic properties(5). While, rabbit VFs are covered with stratified squamous epithelium as seen in human VFs, the three-layered structure of the LP described above is not as clearly defined. Rabbit LP is divided into two layers, SLP and DLP with the vocal muscle extending deeper than the two layers seen in humans (Figure 1). The superficial layer of the LP is comprised primarily of loose ground substances that closely resemble those found in human VFs(16).

1.2 EXTRACELLULAR MATRIX COMPOSITION OF THE VOCAL FOLD

The fibrous (collagen & elastin) and interstitial (fibronectin, decorin and fibromodulin) proteins present within the layers of VF tissue play an important role in maintaining the vibratory mechanics involved with phonation.

1.2.1 Collagen & elastin

Collagen is one of the most abundant proteins in the human body and accounts for approximately 43% of the total protein present within the LP of VFs(17, 18). Collagen fibrils aggregate into large bundles of collagen fibers, and play a structural role in providing strength while maintaining the mechanical properties, shape and organization of the tissue, and directly interacting with cells, to regulate their cell proliferation, differentiation and migration(19). Collagen types I & III have been detected as key components in the VF tissue(17, 20). Type I collagen provides high tensile strength, while type III is found predominantly in tissues that require flexibility and elasticity(21).

Although collagen does not support tissue stretch directly, it provides the strength to partner elastin's stretch and recoil properties in the VF tissue(7, 22). Elastin fibers are composed of the elastin protein and associated fibrillar components such as elaunin and oxytalan. Elastin is an essential component of the VF ECM, constituting approximately 9% of the tissue in comparison to other connective tissues such as skin, where elastin makes up 2-4%(23). Elastin plays an essential role in phonation, with this fibrous protein, appearing as short compact fibers, responsible for tissue flexibility, strength and elasticity.

1.2.2 Interstitial proteins

Interstitial proteins, essential in cell-cell interactions, are collectively seen as protein chains connected to branched polysaccharides. The relationship and ratio of fibrous proteins such as collagen and elastin to the interstitial proteins are essential to VF function, modulating the biomechanical properties and also enhancing the oscillatory characteristics of the tissue. This family of proteins also have strong effects on tissue viscosity, fluid content, thickness of the

LP layers, and also influence collagen fiber population density and size. Interstitial proteins are divided into proteoglycans including HA, decorin and fibromodulin and glycoproteins, such as fibronectin(24, 25).

1.2.2.1 Hyaluronan

HA is a naturally occurring linear polysaccharide comprised of repeated units of D-glucuronic acid and N-acetyl D-glucosamine, and ubiquitously found in the ECM of all tissues, especially mechanically active tissues, including the VF (Figure 2A)(26, 27). HA is negatively charged and interacts with water, acting as a space filler and shock absorber(27-29). These functions are essential to the VF tissue vibratory function. Within the VF, the highest levels of HA are found in the infra-fold, co-localizing with the region where mucosal waves are initiated during phonation. In the absence of HA, studies have shown that there is significant impact on the regulation of tissue elasticity and stiffness, that leads to decreased vibration and VF function(30). In addition to its biomechanical functions, HA, dependent on its chain length, can modulate cellular functions, such as motility, morphogenesis, adhesion, cell survival, apoptosis and metastasis(31-34).

In the ECM, HA synthesis and degradation is strictly regulated, with HA chain half-life differing from less than a day in tissues with rapid turnover, such as the skin, to 2-3 weeks in articular cartilage(35, 36). HA synthesis and polymerization takes place at the inner face of the plasma membrane. Hyaluronan synthase isoenzymes (HAS1, HAS2 and HAS3)(37) use a two sugar nucleotide such as uridine diphosphate, glucuronic acid or UDP N-acetylglucosamine to generate a polymeric chain, which translocates through the plasma membrane into the ECM (Figure 2B)(38-40). HA is degraded by enzymes of the hyaluronidase family (HYAL1-6). HYAL1 and 2 are principally involved in the degradation of HA within somatic cells, including those of the VF. These enzymes work in collaboration, with HYAL2 adhering to HA extracellularly via HA receptors e.g. CD44, cleaving the full-length HA polymer in acidic endocytic vesicles, with HYAL1 further cleaving the HA oligomers using β-exoglycosidases (Figure 2C)(35, 36).

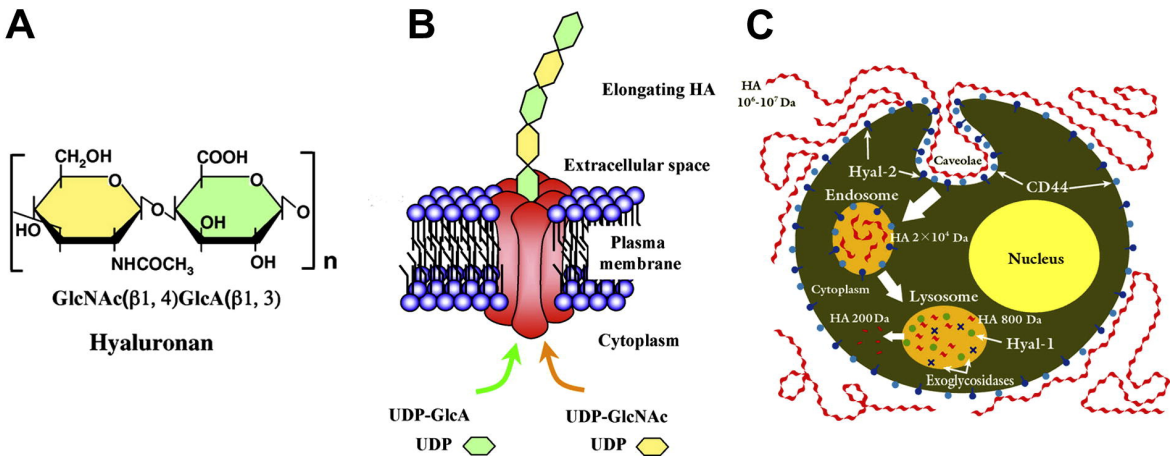

Figure 2: The synthesis and degradation of hyaluronan (HA). (A) HA is composed of repeated units of D-glucuronic acid and N-acetyl D-glucosamine. (B) HA synthesis and polymerization takes place at the inner face of the plasma membrane, with monomeric units added to the chain by members of the hyaluronan synthase family. (C) Hyaluronan degradation occurs through the collaborative action of hyaluronidases (HYAL) found on the plasma membrane surface (HYAL2) and intracellularly (HYAL1) in association with lysosomal bodies. Figure is reproduced with permission from *Dicker et al., Acta Biomaterialia, 2014*, Apr;10(4):1558-70 (26)

1.2.2.2 Small leucine-rich proteoglycans and fibronectin

The presence of small leucine-rich proteoglycans within the VF appear to be co-ordinated by layer and respective function. Decorin, found within the SLP, has an affinity to type I collagen that functions to decrease collagen fiber size in collagen fibril formation and regulate the bundle thickness(41). The core protein is decorated with a single dermatan/chondroitin sulphate chain. In addition to its interactions with collagen, decorin binds to transforming growth factor beta 1 (TGFβ1), a growth factor essential to the healing process and strongly linked to tissue fibrosis if unregulated(42).

Fibromodulin, a keratan sulphate proteoglycan, is found particularly in the intermediate and deeper layers of the VF tissue, close to collagen and elastin(28, 29). Binding to a separate site on the collagen core protein to that occupied by decorin, fibromodulin can limit fibril formation. Strong links between fibromodulin and scarless wound healing have been reported, potentially through its ability to inhibit the activity of TGFβ1(43).

Fibronectin is the most common glycoprotein in the ECM, essential to the wound healing process. It provides support, binding proteins and cells to the ECM. Through the presence of functional domain and cell binding regions, this glycoprotein can influence multiple cellular pathways, including chemotaxis, and can act as a scaffold for stromal cell migration. In the VF tissue, fibronectin is seen principally within the SLP and infrequently in the DLP(44).

1.3 EMBRYONIC STEM CELLS

Embryonic stem cells (ESCs) are pluripotent cells that can go through unlimited self-renewal and maintain the pluripotency to differentiate into any cell type in the body. During mammalian embryogenesis, the fertilized oocyte develops into a multicellular organism. ESCs are derived from the inner cell mass (ICM) of blastocysts after 5-6 days of fertilization. Human embryonic stem cells (hESCs) maintain pluripotency and are strictly regulated by a core set of transcriptional factors such as Oct4, Sox2 and Nanog (45, 46). hESCs have the developmental capacity to form all derivatives of three embryonic germ layers including endoderm, mesoderm and endoderm(47), and can be differentiated into various cell lineages utilizing supplements added into the culture medium(48).

Several animal studies have been reported that use of hESCs differentiated into particular lineages improves tissue functionality. For instance, injection of oligodendroglial progenitors derived from hESCs have been reported to differentiate into neurons and astrocytes following transplantation and improve outcome in spinal cord injuries(49). Likewise injected cardiomyocytes enhance muscle contractile properties of the myocardium after myocardial infarction(50, 51). Similarly, pancreatic β cells derived from hESCs produce insulin and amylin, restoring pancreatic function in mice(52, 53).

To date, three hESC based cell therapies have entered into clinical trials for spinal cord injury, macular degeneration, and type I diabetes(54). With the knowledge that hESCs can differentiate into different lineages such as muscle, epithelium, and cartilage, we hypothesized that these cells could help restore the function of scarred VFs. We have previously injected hESCs into injured rabbit VFs. The results from this study demonstrated tissue regeneration in the VFs, increased viscoelasticity and minimized scarring after 1 month(55).

1.4 MESENCHYMAL STROMAL CELLS

Mesenchymal stromal cells (MSCs), originally termed as mesenchymal stem cells, were first reported by Friedenstein in 1960(56). These bone marrow resident cells, were characterised by their plastic adherence, formation of colonies indicative of self-renewal capacity, and their propensity to differentiate into osteoblasts.

Since then our knowledge of MSCs has grown exponentially. Discovery that these cells are immunomodulatory and key players in the wound healing process has undoubtedly influenced their development as a cell therapy. Since their initial report, stromal cell sources have also been validated in other tissues of fetal and adult origin, including fat, umbilical cord and dental pulp. These findings, and the development of MSC cell therapies from multiple sources, called for more stringent defining criteria, which was outlined by the International Society of Cell and Gene Therapy (ISCT) in 2006. These criteria state that an MSC must be able to demonstrate plastic adherence, self-renewal capacity, possess multipotent capability (cells able to differentiate into mesenchymal lineages; bone, fat and cartilage), express the cell surface markers CD90, CD73, CD105 and human leukocyte antigen (HLA) I, and be negative for the hematopoietic and fibrocyte surface markers CD45, CD34, CD31, CD19 and HLA II(57).

These original guidelines remain limited by the fact that many stromal cell sources possess these phenotypic markers. No consideration is placed on the distinct molecular differences in MSC sources dependent on memory of their tissue of origin. It has therefore become increasingly apparent that in understanding the mode of action of an MSC, consideration has to be placed on its anatomical source. Likewise choosing an appropriate cell source for cellular therapies will be crucial in establishing therapeutic efficacy(58).

1.5 LARYNGEAL DISORDERS

Laryngeal disorders have a significant impact on voice quality, with almost one-third of the adult population experiencing dysphonia at some point during their lifetime(59). This condition is associated with a high degree of morbidity for the patient, impacting communication and the ability to work, leading to unemployment, isolation and depression(59-62). Damage to the vocal ligament (Figure 3) is present in 60-80% patients who suffer with severe dysphonia, strained phonation and reduced VF vibration(2, 63).

Voice quality, pitch or loudness change significantly in vocal disorders, and are associated with other complaints such as vocal fatigue, pre-laryngeal pain, pharyngolaryngeal reflux, throat irritation and throat clearing. Vocal disorders disturb laryngeal function by changing the VF structure (organic disorders) and function (functional disorders). Organic disorders include physical changes in the VF tissue such as edema, or nodules and structural alterations in the larynx due to aging. In addition, problems with central or peripheral nervous system innervation in the larynx can affect vocal mechanisms, leading to disorders such as VF paralysis or spasmodic dysphonia. In contrast, functional disorders are associated with inefficient vocal vibration. The VF structure remains normal, however, there is vocal fatigue and the voice may be characterized by dysphonia or aphonia, ventricular phonation, and diplophonia. Work is ongoing to identify populations at risk for voice disorders, in order to develop early screening procedures(63, 64).

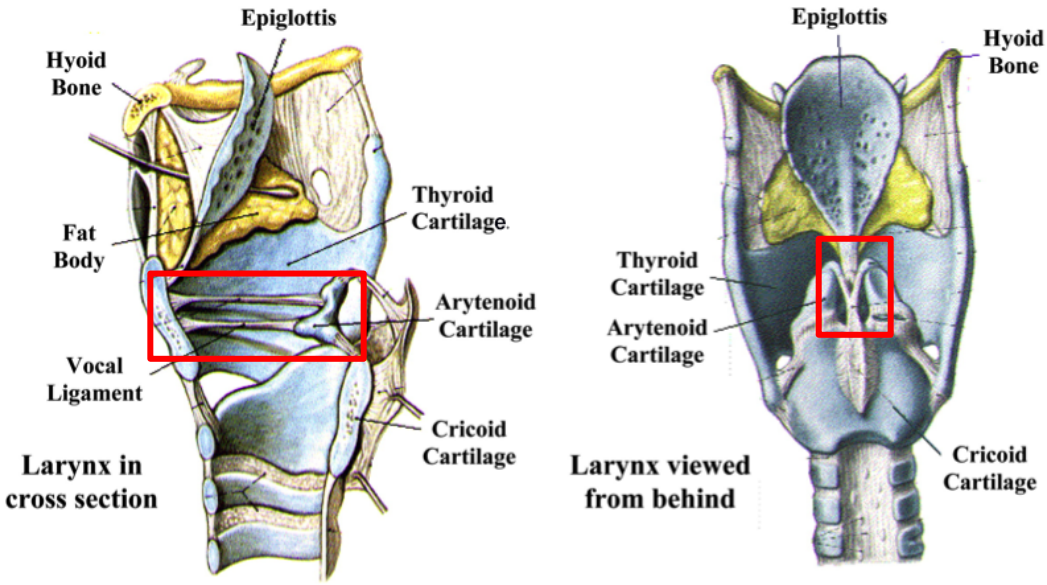

Figure 3: Anatomy of the human larynx. The vocal folds are attached posteriorly to the arytenoid cartilage and anteriorly to the thyroid cartilage. This can be seen from the two cross-sectional viewpoints of the larynx above (location of the vocal folds are highlighted by red boxes on each image). The figure is adapted with permission from *Miri AK, Journal of Voice, 2014, Nov;28(6):657-67 (65).*

To identify the effect of age and gender on individuals who develop functional or organic voice disorders, Van Houtte's team conducted a retrospective investigation on laryngeal pathology diagnosed using videostroboscopy(66). The findings from this study indicated that functional voice disorders are the most common (30%), with no cases seen in childhood. This was followed by VF nodule disorders (15%), and pharyngolaryngeal reflux disorders (9%). The pathologies were significantly higher in females (63.8%) than in males (36.2%). For professional voice users, functional dysphonia occurred in 41% of individuals, with teachers mainly affected(66). The majority of these pathological findings have been similarly demonstrated in a further study conducted on individuals between the ages of 45-64(63).

The most commonly diagnosed changes to the voice come with age due to presbyphonia or VF atrophy. The VF tissue in elderly individuals undergoes significant changes leading to severe hoarseness, that is difficult to treat(67). VF scarring in the large spectrum, which may include VF atrophy, can be the result of sulcus vocalis, postsurgical scarring, phonotrauma, or direct trauma (radiation or reflux disease). Although the causes of VF scarring are distinct, the phonatory outcomes are similar in all conditions. Loss of phonation due to scarring results in mucosal wave impairments with loss of pliability and incomplete glottal closure(68). Pliability of the VF is significantly affected in this condition and presents as incomplete glottal closure due to thinning or bowing of the vocal cords. Sulcus vocalis is a long, thin groove running along the edges of the SLP of the VF. This defect can be restricted to the SLP or penetrate deeper into the DLP and muscle(69) (Figure 4).

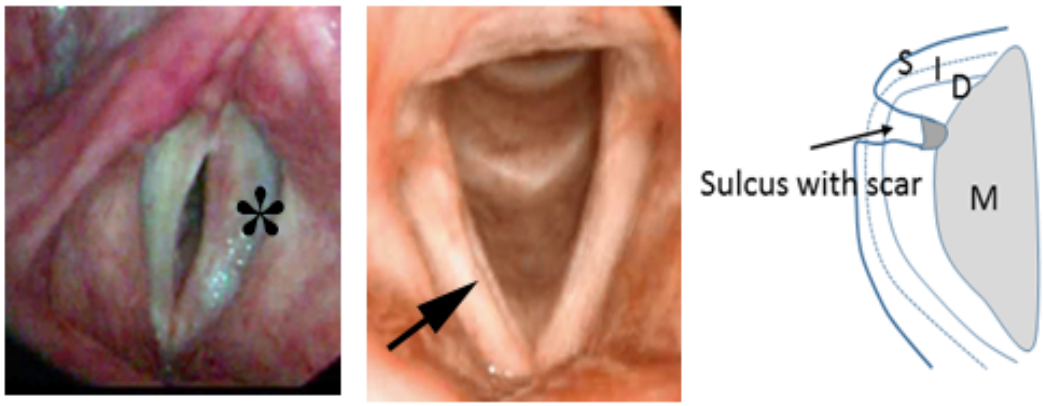

Figure 4: Images taken using videostroboscope highlighting (A) scarring in the left VF (*) and slight atrophy in the right VF. (B) Bilateral sulcus vocalis, most evident in right VF (arrow). (C) Schematic representation of sulcus vocalis. Abbreviations: S - superficial LP; I -intermediate LP; D – deep LP; M – muscle.

1.6 VOCAL FOLD SCARRING

Damage to the VF tissue that disturbs ECM organization can change the viscoelastic properties of the VFs, resulting in hoarseness and poor voice quality. Severe damage due to direct injury or external trauma of VF tissue may lead to permanent pathological changes, with scar formation, inhibition of mucosal wave formation and dysphonia(70). Systematic approaches have been utilized to characterize the role of the ECM in VF wound healing and scarring in various animal models, including bovine, porcine, ovine and lapin systems, have been developed to examine the wound healing properties of VF tissue(71-76). The relationship between ultrastructural changes in the ECM, especially collagens, elastin and HA, as well as, the corresponding viscoelastic properties remains poorly understood.

Fibrous scar tissue consists primarily of type I collagen, with stiffness of the scarred VF dependent on the collagen content and organization. Studies have demonstrated that, post-injury, increased levels of type I procollagen are deposited in the SLP, and that the collagen composition is less dense and becomes disordered compared to healthy tissue. Similarly, injury results in a loss of elastin fiber density and organization. As the scar tissue develops with time procollagen levels increase, and although elastin levels are restored within the tissue, their organization, much like that of the collagen, remains unstructured, thereby impeding function(5, 71).

Scar tissue is not solely composed of type I collagen and elastin. Recent research in the field has suggested a need to consider other ECM molecules including HA, fibronectin, decorin and other collagen types that can also regulate tissue viscoelasticity and function. Levels of HA may also influence wound healing and scar formation. Changes in HA density and distribution between scarred and healthy VFs have been investigated in both rabbit and canine models. Levels of HA were lower in injured VFs during the initial stages of wound repair compared to healthy controls. These data suggest that adding HA in the early stage of wound healing, or in the clinical situation, after resection of the scar tissue, may have an beneficial impact on decreasing the incidence of VF scar formation(77).

Both decorin and fibromodulin have been reported to limit scarring within the injured VF through their ability to actively bind and inhibit TGFβ1, which up-regulates collagen synthesis by stromal fibroblasts, and has been directly linked to tissue fibrosis by converting fibroblasts into contractile, alpha smooth muscle actin (αSMA) expressing cells(29).

Fibroblasts secrete an extensive network of fibronectin and type III collagen. Fibronectin has a stronger affinity to type III collagen than any other collagen type, which forms a primary constituent of granulation tissue. Over time, granulation tissue begins remodeling by decreasing fibronectin and type III collagen, as well as simultaneously increasing the content of type I collagen to later contribute to matrix organization(78). It was previously reported that high levels of fibronectin are present in hypertrophic and chronic wounds, and that the persistence of elevated levels of fibronectin in the ECM can lead to fibrosis during the healing process(79). This has been evidenced in scarred rabbit VFs 2 months post-injury(78, 80, 81).

1.7 VOCAL FOLD WOUND HEALING

In general, wound healing is a dynamic and complex process consisting of a series of co-ordinated events between diverse immunological and biological systems. It commences with bleeding and coagulation, and evolves into acute inflammation, cell migration, proliferation, differentiation, angiogenesis, synthesis and remodeling of the ECM and re-epithelialization. These complex events can be divided into four overlapping time-dependent phases 1) hemostasis 2) inflammation 3) proliferation and 4) remodeling (Figure 5).

1.7.1 Hemostasis

Coagulation and hemostasis take place immediately after injury, to prevent exsanguination. The dynamic balance between endothelial cells, thrombocytes, coagulation and fibrinolysis regulates hemostasis and the reparative process. After injury, blood enters the injury site, and platelets begin to aggregate with collagen, and other ECM components. This initiates the formation of a blood clot composed of fibronectin, fibrin, vitronectin and thrombospondin. The platelets trapped within the clot provide a provisional matrix for cell migration in the later phases. This triggers the release of growth factors and cytokines, such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), epidermal growth factor, vascular endothelial growth factor (VEGF), and TGFβ1. These molecules act as promotors of the wound healing cascade, and attract immune cells (neutrophils, monocytes, lymphocytes) to the wound site. Cytokines released from immune cells including tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukin (IL)-1β, PDGF, bFGF and TGFβ1 further modulate fibroblast activation in the wound bed(82, 83).

1.7.2 Inflammatory phase

This phase begins after immune cells reach the site of injury. Neutrophils are the primary effector cells present 24 hours after injury. They are guided to the wound site by chemokines and other chemotactic agents such as TGFβ1, formyl-methionyl peptides from bacteria, and platelet products. These cells are implicated in the clearance of foreign material and bacteria by phagocytosis(83). Monocytes, which are the precursors of tissue macrophages appear in the wound after 48-72 hours and continue the process of phagocytosis. Monocytes are attracted to the wound by a myriad of chemoattractive agents, such as PDGF, TGFβ1, elastin and collagen breakdown products(84). Lymphocytes enter the wound 72 hours after injury by secretion of IL-1β, complement products and immunoglobulin G (IgG) breakdown products. IL-1β plays a crucial role in the later phase of collagen remodeling and synthesis

of ECM components, and their degradation(85-87). Further discussion surrounding the role of the innate immune response in the wound healing cascade can be found in section 1.8.

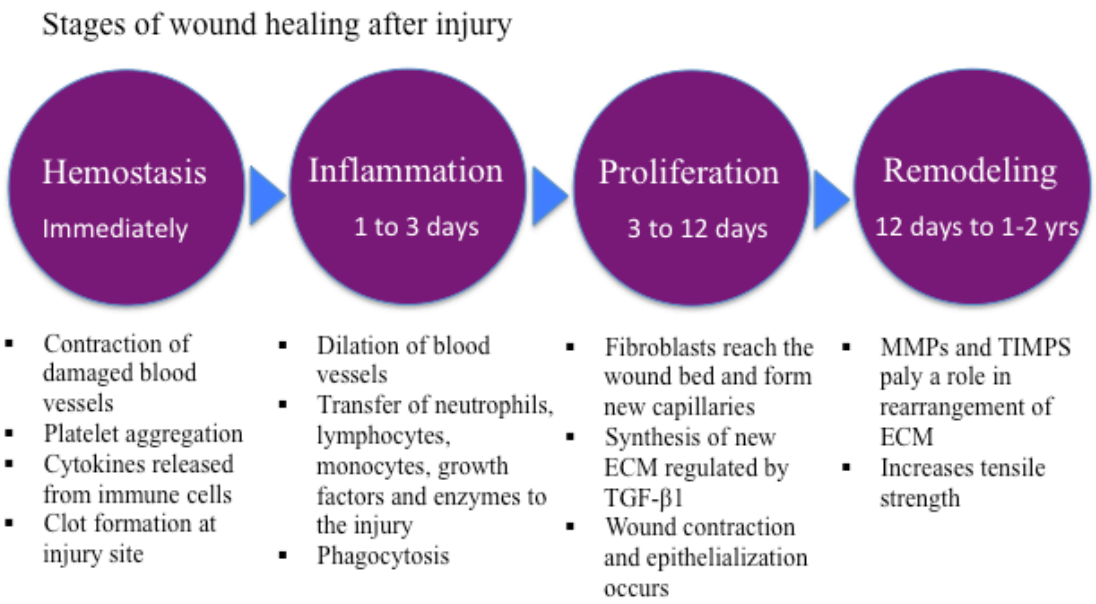

Figure 5: The stages of wound healing. A schematic representation of the stages of the wound healing cascade and their associated key events.

1.7.3 Proliferative phase

The proliferative phase begins approximately 3 days after injury, and continues for 2 weeks, involving fibroblast activation, migration and the production and deposition of ECM, resulting in the formation of granulation tissue. In response to increased levels of TGFβ1 in the wound, fibroblasts change their phenotype into myofibroblasts, expressing αSMA. These myofibroblastic cells are involved in synthesizing and depositing ECM components. In this phase, fibroblasts enter the fibrin clot and release various matrix metalloproteinases (MMPs), which degrade the clot, replacing this with ECM components such as collagens I, III, IV and VII and glycoproteins such as fibronectin, proteoglycans, and HA. This complex matrix supports angiogenesis and re-epithelization(88).

Myofibroblasts exhibit contractile properties and play a crucial role in the contraction and maturation of granulation tissue(89). When newly synthesized ECM forms, the cellular levels including myofibroblasts are decreased markedly around the scar tissue. This reduces stiffness, with the release of mechanical stress, and also decreases αSMA expression and contractibility of myofibroblasts. The excessive or redundant fibroblasts are eliminated by apoptosis or phagocytosis either by macrophages or neighboring cells(90-92). The internal connective tissue shrinks in size, and allows fibroblast interactions with the ECM. This process is highly regulated by PDGF, TGFβ1, and bFGF(93, 94). Moreover, the growth of capillaries is inhibited, leading to a decrease in blood flow and metabolic activity at the wound site. Eventually the mature scar tissue will consist of a reduced number of cells, and capillaries, and will display improved tensile strength(95, 96).

1.7.4 Remodeling phase

After formation of the scar tissue within the wound, the remodeling phase begins and may last for 1-2 years. During this time, proteolytic enzymes, MMPs and their inhibitors, tissue inhibitors of metalloproteinases, play an important role in regulating ECM synthesis and

matrix remodeling. This phase is important in reversing tissue fibrosis, and restoring tissue architecture by maintaining the balance between degradation and synthesis, resulting in collagen remodeling and reorganization, and decreased levels of fibronectin and HA(86, 97).

1.8 INNATE IMMUNE RESPONSE IN WOUND HEALING

The innate immune system is the first line of defense in the wound healing process. It functions rapidly to eliminate the spread of infection. Its non-specific actions in the clearance of harmful agents is dependent on pathogen associated molecular patterns or damage-associated molecular patterns through the toll-like receptor (TLR) system. The cells and molecules involved in this system are principally macrophages, neutrophils, mast cells, eosinophils, interferons, interleukins, anti-microbial peptides and proteins and acute phase proteins. These cells display various characteristics complementing each other's functions by the production of inflammatory mediators through phagocytosis, production of reactive oxygen species, nitric oxide and antigen presentation(98, 99). Monocytes are the prominent cells in the inflammatory response that allow co-ordination between the innate and adaptive immune responses to improve host defense against pathogens.

1.8.1 Monocytes and macrophages in tissue repair

Monocytes are found in the blood, bone marrow, and spleen, and are recruited to the injury site during the homeostasis phase. They act as effector cells in their own right and as progenitors of macrophages and dendritic cells. Initially monocytes were characterized by their expression of CD14, and later the additional detection of CD16. Cell surface markers and functional activities distinguish heterogeneous types of monocytes, such as inflammatory (classical) or anti-inflammatory (non-classical) subsets. Human classical monocytes are CD14+CD16-, intermediate subsets express both CD14 and CD16 (as well as an increased level of HLA II), and non-classical monocytes are CD14-CD16+. The three subsets vary in expression of C-C chemokine receptors (CCR), adhesion molecules, and differentiation properties(100, 101). For instance, CD14+CD16+ monocytes express CCR5 and CD14+CD16- monocytes express CCR2(102).

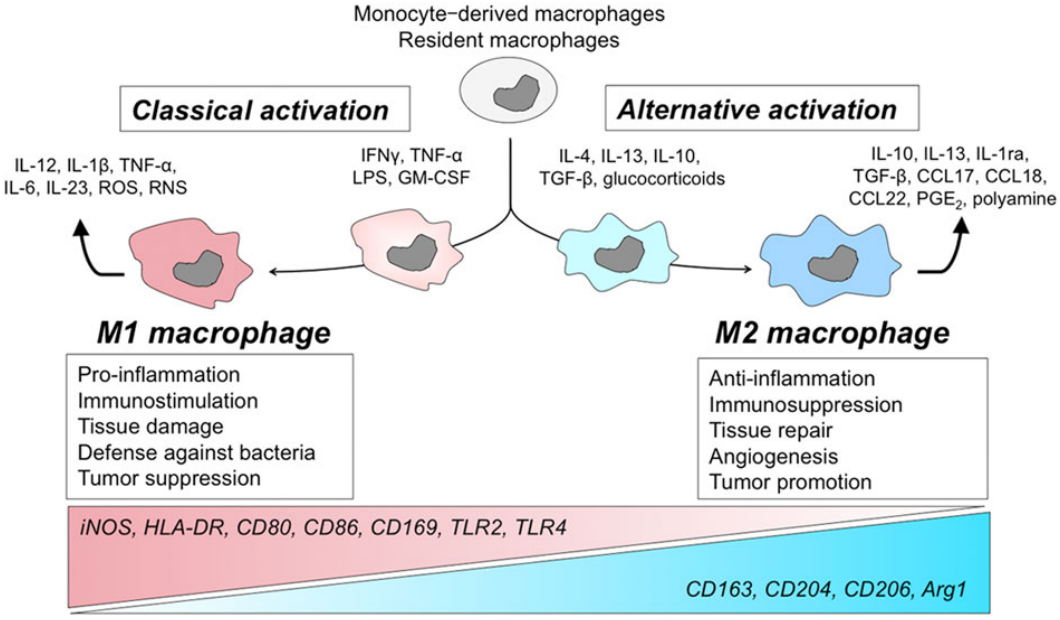

Figure 6: Schematic representation of monocyte differentiation into pro-inflammatory (M1) and anti-inflammatory (M2) macrophages. Figure reproduced with permission from *Takeya & Komohara, Pathology International, 2016, Sep;66(9):491-505(103)*

Macrophages can respond to endogenous stimuli that are generated immediately after injury or infection. In addition, macrophages can also respond to signals coming from antigen-specific or adaptive immune cells. In response to these environmental stimuli, macrophages demonstrate multidirectional polarization. The subtypes of polarized macrophages are classified as classically activated or inflammatory macrophages (M1) and alternatively activated or anti-inflammatory macrophages (M2). In response to pro- inflammatory cytokines such as TNF-α, IFN-γ and lipopolysaccharide (LPS), the macrophage activates and differentiates into an M1 phenotype. This subset of macrophages encourages inflammation by releasing pro-inflammatory cytokines (IL-12, IL-1ß, TNF-α, IL-6 and IL-23), as well as, reactive oxygen species and nitrogen intermediates, which can stimulate other immune populations. M1 macrophages are characterized by their expression of several markers including inducible nitric oxide synthase (iNOS), HLA-DR, CD80, CD86, and TLR 2 and 4 (Figure 6). Up-regulation or failure to resolve the M1 macrophage response can lead to further damage in the tissue through activation of pathogenic T helper 17 cells(103, 104).

M2 anti-inflammatory macrophages can be divided into 4 main subtypes, M2a-d, based on the applied stimuli. The M2a subset is induced by IL-4 and IL-13, M2b by immune complexes and LPS, M2c by glucocorticoids and TGFβ1 and M2d by adenosine and IL-6(105, 106). Collectively these macrophages are considered as anti-inflammatory mediators, stimulating wound repair by secreting factors such as IL-10, IL-13 and TGF-ß1. M2 macrophages also stimulate phagocytosis of neutrophils, and promote the migration and proliferation of stromal fibroblasts, epithelial and endothelial cells to promote tissue repair, epithelialization and neovascularization(107, 108).

Mature tissue macrophages express various cell surface receptors. Scavenger receptor is one type of group of pattern recognition receptors that has a broad range of ligand binding. Scavenger receptor cysteine-rich (SRCR) are a subgroup of the scavenger receptor family. The SRCR family is divided into group A and B based on the spacing pattern differences between their cysteine residues. Group A comprises of 6 cysteine residues, whereas, group B consists of 8 cysteine residues. The molecules included in group A are class A scavenger receptor I, Mac-2 binding, macrophage-associated receptor with collagenous structure, complement factor I and enterokinase(109).

The transmembrane glycoprotein CD163 (130 kDa) belongs to the class B SRCR family, along with other molecules including CD5, CD6, SPα and gp-340. CD163 is expressed on a sub-population of mature tissue macrophages (strongly associated with M2a and M2c macrophages) and has been described in humans, marmosets and pigs (110-112). It is also described as an ED2 antigen (mature tissue macrophage) in rat(113). Previously it was reported that increased levels of CD163 expression were found in mature tissue macrophages including liver kupffer cells, red pulp macrophages in the spleen, macrophages in the thymus and resident bone marrow macrophages(114). Typically, CD163 is considered to be a marker of anti-inflammatory macrophages, induced by glucocorticoids and anti-inflammatory mediators, such as IL-6 and 10(115, 116) (117, 118). However, reports have also demonstrated that IFN-γ and LPS can upregulate CD163. These seemingly opposing stimuli, illustrate the roles that CD163 plays in both suppressing inflammation, as well as, in the scavenging of damaged and dead cells/cellular debris(119).

Both M1 and M2 macrophages play an important role in myofibroblast activation during fibrosis. Following the build-up of excessive inflammation in tissue, M1 macrophages can initiate the pro-fibrotic process by releasing various cytokines that recruit fibrocytes via chemokine (C-C motif) ligand 2 (CCL2), which then migrate to sites of inflammation and

promote the proliferation of myofibroblasts through release of MMPs that degrade the ECM(120, 121). When the acute phase of inflammation comes to an end, M2 macrophages are generated by stimulation of the aforementioned cytokines or via phagocytosis of apoptotic bodies, triggering anti-inflammatory processes through the release of IL-10, TGFβ1, arginase and heme oxygenase-1, aiding the transition to the proliferative phase of the healing process(122, 123).

1.9 THE ROLE OF MESENCHYMAL STROMAL CELLS IN WOUND HEALING

Several studies have shown that MSCs can migrate to injured tissue sites in response to the chemokine stromal cell-derived factor-1 (SDF-1), which interacts with its receptor C-X-C chemokine receptor 4(124-127). Although the role of endogenous MSCs in the VF has not been explored with respect to healing responses, knowledge regarding their ability to modulate the different stages of the healing cascade have been hypothesized from other tissues (Figure 7).

In generic terms, during the inflammatory phase of the healing response, when MSCs are exposed to pro-inflammatory cytokines (TNF-α, IFN-γ and IL-1β), they become activated, resulting in the up-regulation of chemokine receptors such as macrophage-derived chemokine receptors (CCR2, CCR3, and CCR5), which can mediate their migration to sites of injury(128-132). They furthermore, modulate their expression of chemokines and cytokines, such as IL-6, and iNOS, which can directly modulate both innate and adaptive immune subsets (including upregulating of regulatory T cells) to decrease the secretion of pro-inflammatory cytokines in favor of anti-inflammatory cytokines such as IL-10 and IL-4. These immunomodulatory properties and ability to sense environmental change make MSCs a potentially valuable therapeutic option for wound healing(133).

MSCs have been shown to play a role in the recruitment of both monocytes and M2 macrophages within inflamed tissue by the secretion of factors such as CCL1, CCL3, CCL12, chemokine (C-X-C motif) ligand 2 and macrophage colony-stimulating factor (M-CSF)(134). Some studies have reported that MSCs can suppress tissue inflammation through skewing of M1 pro-inflammatory macrophages, or via direct induction of monocytes, to an M2 phenotype by cell-cell contact or by MSC secretion of prostaglandin E2 (PGE2), IL-6 and indoleamine 2,3-dioxygenase (IDO)(135-137). This switch to an M2 phenotype is accompanied by activation of cytokines and growth factors that have anti-fibrotic properties including hepatocyte growth factor (HGF). The growth factor HGF can limit myofibroblast differentiation, and regulate fibroblasts in the wound to produce a more organized and regulated ECM akin to that of the native tissue(138, 139). Fibroblasts respond to HGF by specifically down-regulating the expression of TGFβ1 and type III collagen by nuclear exclusion of SMAD3, a transcriptional factor regulating various pro-fibrotic factors including TGFβ1. HGF also promotes the up-regulation of collagenases, MMPs 1, 3 and 13, in fibroblasts and M2 macrophages, hence promoting the turnover of ECM, and breakdown of scar tissue.

During the proliferative phase of wound healing, MSCs secrete pro-angiogenic factors (VEGF, angiogenin and leptin), which increase the density of microvessels and improve tissue vascularization within the wound. MSC secreted growth factors, such as insulin-like growth factor 1, PDGF, TGFβ1, SDF-1 and HGF, also promote the migration and proliferation of vascular endothelial cells and fibroblasts(140, 141).

During the remodeling phase, MSC paracrine signaling may also promote an anti-scarring effect. MSCs secrete large amounts of VEGF and HGF, playing a role in maintaining higher ratios of the fetal, scarless healing form TGF-β3 to scar inducing TGFβ1(142).

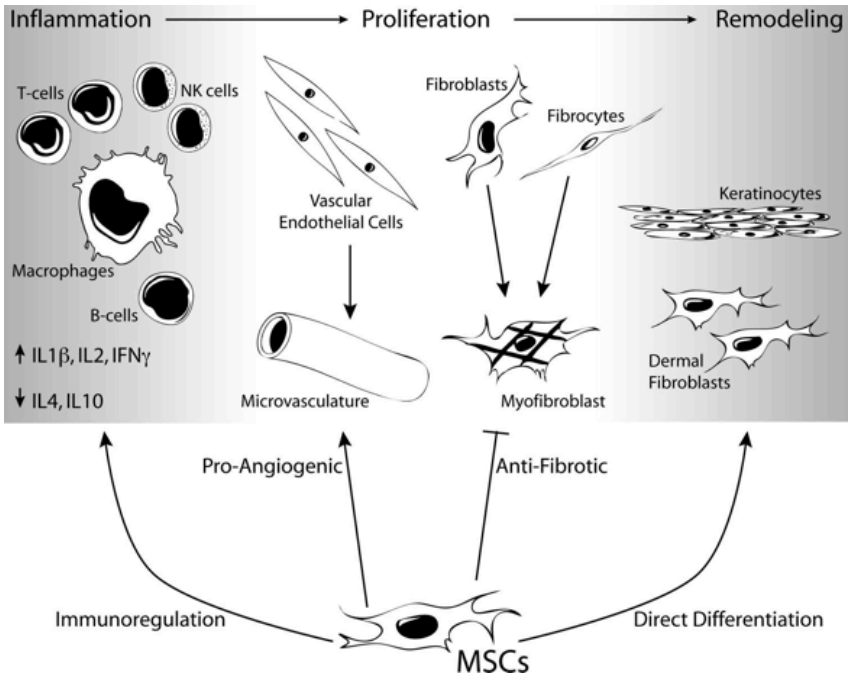

Figure 7: The multi-faceted role of the mesenchymal stromal cell (MSC) in tissue repair. MSCs have been exploited in tissue engineering and therapeutic applications for their immunoregulatory, anti-fibrotic and multipotent properties. Figure reproduced with permission from *Jackson et al. Stem Cells Translational Medicine, 2012, Jan;1(1):44-50(143).*

1.10 TREATMENT STRATEGIES FOR VOCAL FOLD DISORDERS

Treatment options for the aforementioned VF disorders are organized under three main approaches.

Voice therapy is an important part of treatment for various vocal disorders. This can take the form of:
A) Direct or physiologic voice therapy, which directly alters the physiology of the vocal mechanism by practicing voice therapy methods such as the accent method(144), cup bubble blowing(145), expiratory muscle strength training(146), Lee Silverman voice treatment(147), stretch and flow phonation, and vocal function exercises(148).

B) Indirect or symptomatic voice therapy treatment is aimed at modifying the deviant vocal symptoms or perceptual voice components using a variety of techniques. Deviant symptoms include changes in the voice pitch, volume and breathy phonation. Voice facilitating techniques such as amplification, auditory masking, chant speech, confidential voice therapy, semi-occluded vocal tract exercises, straw phonation and twang therapy are used to improve voice quality. The above treatments are selected based on the type and severity of the symptoms and the patient's experience(149-153).

Medical treatments, such as proton-pump inhibitors used for anti-reflux, can help treat patients suffering with reflux laryngitis. Type A botulinum toxin, used to treat muscle disorders, has also been shown to be beneficial in the treatment of voice disorders caused by muscle spasms or spasmodic dysphonia(154). **Surgical treatments** have increased dramatically in recent years for the treatment of voice disorders. Phonomicrosurgery has been valuable in the removal of lesions or abnormalities that inhibit VF vibration(155), as well as, laryngeal framework surgery, which involves surgical manipulation of the larynx framework in order to enhance VF closure and improve vibration(156).

1.10.1 Treatment strategies for vocal fold scarring

Voice therapy for patients with VF scarring has demonstrated to be less effective in improving voice quality, while other options to resect scar tissue by surgery usually has limited effect and may even worsen the issue(157, 158). There is currently no effective treatment available to treat VF scarring. This complex condition, treated in clinical settings, produces inconsistent and suboptimal results, with the need for further understanding into the pathology and development of novel treatment options.

Preclinical research for the development of novel treatments for VF scarring is mainly focused on two approaches: 1) prophylactic treatment or preventive approaches for attenuation of scar formation after injury of the VF tissue and 2) treatment of chronic scar tissue for restoration of vibration function.

Prophylactic treatments are based on the concept that the acute healing phase serves as a critical period during which therapeutic intervention may attenuate eventual scar formation. From a clinical perspective, this approach would involve excision of the scar tissue to induce a wound healing response. In the acute wound, resident fibroblasts start to synthesize ECM 2-3 days after injury(159). Prophylactic therapeutic interventions given at time of injury, in preclinical animal models, include MSCs, vitamin A, corticosteroids, HA-based biomaterials (discussed further below and in section 1.12) and decellularized scaffold materials.

Previously, we have published the benefit of locally injecting human bone marrow MSCs (BM-MSCs) at the time of VF injury in a rabbit model. Three months post-injury there was a significant reduction in type I collagen deposition, reduced LP thickness and an improvement in viscoelastic properties compared to the saline treated control group(160, 161). Vitamin A (topical retinoic gel) has also been used at the time of injury in a rabbit VF model, resulting in less collagen deposition in the treatment groups compared to the saline controls(162), as well as playing an important role in the repair and maintenance of the VF epithelium(163, 164).

Corticosteroids, used primarily for their ability to dampen inflammation, have been suggested to also assist in wound healing, resulting in a significant reduction in collagen deposition post-injury, and inducing fibroblasts to begin synthesizing ECM with collagen, HA and fibronectin deposition most prominently at day 3-5 days post-injury in rabbit VFs (159, 165). The data from these studies show a positive therapeutic effect with corticosteroid delivery during the acute phase of wound healing. Further work is required to evaluate this beneficial effect on later stages of wound healing.

Treatment of chronic VF scarring remains a challenge clinically. Several new approaches are currently under development, including cellular therapies (discussed further in sections 1.11 and 1.13), local administration of growth factors and the use of scaffolds. In sulcus vocalis patients, autologous fascia transplantation has been reported with improvements in

phonation, mucosal wave function and glottal closure pattern(166-169). The data from these example studies demonstrates that implantation of fascia is beneficial in the treatment of this cohort of patients.

Various animal models have investigated the use of pro-healing growth factors to enhance the wound healing process. bFGF is one such example, due to its known role in modulating stromal fibroblast proliferation and matrix production. Six months post-injection the authors report a reduction in tissue contraction, higher abundance of glycosaminoglycans (GAG), increased vibratory amplitude and a lower phonation threshold compared to saline treated control animals(72). The same group reported findings from their clinical study, suggesting significant improvement in glottal closure, acoustic and aerodynamic parameters from VF atrophy patients treated with bFGF(170).

Scaffold materials have been used extensively by otolaryngologists for the treatment of patients with VF defects. Historically scaffold/biomaterials have been used to push the affected VF towards the midline, allowing for closer physical proximity during phonation. Synthetic materials such as silicone, polytetrafluoroethylene-teflon and polyhydroxyethylmethacrylate have been replaced with biodegradable materials, such as collagen matrix or fat, which also have the added advantage of being able to improve viscoelastic properties to that comparable with healthy VFs(171, 172). HA- and collagen-based hydrogels are the most common, both being major components in the native ECM of VF tissue. Bovine collagen has been used for the treatment of glottal insufficiency, but concerns over xeno-reactivity has led to a switch to autologous and micronized AlloDerm(173, 174). Commercially available composite materials including Gelfoam™ paste(175) Cymetra™(176), calcium hydroxylapatite(177), and HA derivatives such as Restylane™ and Hylaform™(178) have also been used in the clinic to enhance VF function. Hydrogel-based delivery of the anti-fibrotic growth factor, HGF, has also been reported in a canine model of VF injury, with data indicating that 3 months after treatment there was reduced PTP, decreased type I collagen, improved mucosal wave excursion and increased HA abundance compared to the saline treated control group(179). Similarly, pirfenidone delivery has also been considered, exhibiting both anti-inflammatory and anti-fibrotic effects *in vivo* and *in vitro*(180). New approaches for the treatment of chronic scarring are also being developed using PGE2. This bioactive lipid is a key anti-inflammatory mediator and additional exhibits anti-fibrotic effects through modulating the TGFβ1 signaling pathway, leading to de-differentiation of myofibroblasts into fibroblasts(181, 182).

1.11 PRE-CLINICAL DEVELOPMENT OF MSC THERAPY FOR VF SCARRING

A surgical and medical treatment to diminish VF scar formation has been met with limited success. Cell therapy is therefore being considered as a potential regenerative approach. To date, cells such as autologous and allogeneic MSCs including adipose derived stromal cells (ADSCs)(183-185), fibroblasts(186, 187), ESCs(55), and induced pluripotent stem cells(188) have been used in pre-clinical animal models with the aim of resolving VF tissue damage and reducing scar formation with varying results.

We have previously reported in a rabbit model of VF injury that local injection of human BM-MSCs at the time of injury, results in a reduction in levels of type I collagen, reduced LP thickness, and improved viscoelastic properties and histology compared to injured controls at 1-3 months(160, 161). Our team have further demonstrated that human BM-MSCs injected into the rabbit VFs nine weeks post-injury also successfully reduced LP thickness and restoration of viscoelastic shear properties comparable to uninjured animals(189). Our

findings have been validated in further models, including the rabbit, and with other sources of MSCs including ADSCs (reports summarized in table 1).

Supporting studies have investigated in detail ultrastructural changes in fibrous components of the VF ECM post-MSC injection, demonstrating improved collagen structure (thinner fibrils and fibers) and organization, as visualized via atomic force microscopy(190). Effects of MSC delivery have also been reported on scarring with relation to endogenous stromal fibroblasts. In a canine model, autologous BM-MSCs injected into injured VFs improved tissue regeneration(184), and laryngeal mucosa mesenchymal stem cells have been reported to transition myofibroblasts into fibroblasts(191). Likewise, autologous ADSCs injected into injured rabbit VFs, demonstrated a reduction in inflammation and fibrosis compared to controls(192).

Hydrogels and scaffolds, as discussed in section 1.10, have also been combined with MSCs in developing novel therapeutics for VF scarring. Rabbit VFs have been transplanted with a composite gel composed of small intestine submucosa (a collagenous ECM material derived from the submucosal layer of porcine intestine) and rabbit MSCs, which aided in improving MSC survival for up to eight weeks *in vivo*, with a reported increase in HA accumulation within the ECM and a reduction in scarring(193). Atelocollagen sponge has also been combined with autologous canine BM-MSCs and injected into the sub-epithelial pockets of scarred VFs. The results demonstrated increased levels of HA, decreased collagen deposition and an improvement in mucosal vibration compared to the untreated group(194). The use of HA-MSC hydrogels has been discussed further in section 1.12.

Table 1: Summary of pre-clinical *in vivo* studies conducted investigating the potential of stem and stromal cell populations in promoting vocal fold healing. Abbreviations: ADSC – adipose derived stromal cell; BM-MSC – bone marrow mesenchymal stromal cell; ECM – extracellular matrix; ESC – embryonic stem cell; HA – hyaluronan; HGF – hepatocyte growth factor; NA – not applicable

Model	Graft type	Injected cells	Associated materials
Lapin	Xeno	BM-MSC(160, 161, 189, 195)	NA
Lapin	Xeno	BM-MSC	Porcine gel (193)
Canine	Auto	BM-MSC/ADSC (196)	NA
Canine	Auto	BM-MSC	Atelocollagen (184, 194)
Rat	Xeno	BM-MSC(197)	NA
Rat	Allo	BM-MSC/ADSC(198)	NA
Rat	Xeno	BM-MSC	ECM(183)
Lapin	Auto, Xeno	ADSC(160, 185, 192, 195)	NA
Lapin	Auto	ADSC	Collagen or HA(199)
Lapin	Xeno	ADSC	Alginate-HA hydrogel(200)
Lapin	Auto	ADSC	HA and HGF(201)
Lapin	Allo	ADSC	Fibrin hydrogel(202)
Lapin	Allo	ADSC	HA(203)
Canine	Auto	ADSC	Atelocollagen(204)
Canine	Auto	ADSC(205)	NA
Canine	Allo	Laryngeal MSC(191)	NA
Lapin	Xeno	ESC(55, 206)	NA

1.12 HA DELIVERY OF MSCS

As discussed in section 1.10.1, HA has been used safely in laryngology for the treatment of glottal insufficiency and for temporary augmentation in VF defects such as paralysis and atrophy. HA based hydrogels are biocompatible, and offer biomechanical advantages in VF treatments due to their viscoelastic and shock-absorbing capabilities(207). Their resorption rate can be modified based on the mode of chemical crosslinking used in gel formation, which allows adaptation for a variety of conditions as both a scaffold and cell/growth factor delivery vehicle(208, 209). These properties make HA hydrogels an ideal option for delivering MSCs into the injured VF. When BM-MSCs are encapsulated within Extracel™, they maintain high viability leading to an increase in their survival (30 days post injection) and proliferation(210). In addition to enhancing cell survival, surrounding the cells with a HA rich environment encourages the production of an ECM profile more favorable for wound healing.

The use of HA as a delivery vehicle for MSCs, of various sources, has been reported previously for VF injuries. ADSCs combined with HA gel before injection into injured rabbit VFs, demonstrated beneficial results, with the collagen contents decreasing and improving organization with remodeling after 12 months(199). Autologous fibroblasts encapsulated in a HA scaffold have also been suggested to improve rabbit VF structure and function(211).

Likewise, rat BM-MSCs encapsulated in Extracel™ have also been reported to give a superior treatment outcome compared to BM-MSCs alone after one month in a rat model of VF injury(183). Injured rabbit VFs injected with BM-MSCs in HyStem® also reported minimal inflammation, improved viscoelasticity and changes in gene expression consistent with desirable ECM remodeling including lower levels of fibronectin, collagen (types I, II, and III), TGFβ1, αSMA, IL-1β, IL-17β and TNF-α compared to injury only animals, indicating that MSCs may expedite VF tissue repair after injury(212). Injectable HA has also been used in combination with other scaffold materials, including alginate. HA/mildly cross-linked alginate hydrogel has been used in combination with ADSCs demonstrating improvements in VF architecture, with decreased collagen deposition and improvement in the viscoelastic properties(200).

1.13 CLINICAL USAGE OF MSCS IN THE TREATMENT OF VF SCARRING

Clinical MSC therapy has been proven to hold great potential for innovative intervention in the treatment of various diseases and disorders. *In vitro,* MSCs have demonstrated an important role in the inhibition of immune cell activation(213, 214), and within clinical studies this has been recapitulated, with MSC treatment reducing inflammation and exerting positive therapeutic effects on immune-mediated diseases such as steroid-refractory graft-versus-host disease(215, 216) . The majority of these clinical studies have been conducted using intravenous delivery of MSCs (both autologous and allogeneic) with minimal side effects reported for BM-MSCs(217) .

Trials using local injection of MSCs in clinical applications have increased over the last decade. Within the clinical setting for VF scarring, studies are still within their infancy. To date there is only 1 published case report conducted on a patient suffering with severe dysphonia at the time of treatment. The patient, who had already undergone 2 laryngeal phono-surgeries, had marked dysphonia. Autologous adipose derived stromal vascular fraction was injected into both scarred VFs. Final evaluation after 12 months demonstrated an improvement in vocal function, stable phonation with no breathiness. In addition, significant improvement in VF vibration was also observed. No severe side effects were observed due to cell administration(218). This research group also completed a phase I trial in 2018, in 8 patients using the above approach, but the data remains to be reported (ClinicalTrials.gov ID: NCT02622464).

Basic knowledge regarding the safety of local MSC administration has been gleamed from clinical studies in other indications; for example, intradiscal implantation of autologous BM-MSCs has been used in patients suffering from degenerative disc disease. Significant improvements were observed in overall subjective symptoms such as reduced pain, increased function and reduced disc bulge size in the majority of the patients in the 6 years of follow-up study(219). ADSCs in HA hydrogel have also reported to significantly improve disc function in patients with chronic discogenic lower back pain(220). Similar positive findings have also been reported regarding the use of local administration for tissue repair responses, including the closure of fistulas in Crohn's disease patients with, as previously reported, no negative side effects of MSC treatment(221). Intramuscular injection of allogenic MSCs have also been safely employed for the treatment of digital ulcers in systemic sclerosis autoimmune disease(222).

2 AIMS OF THE THESIS

hESCs have the capacity of self-renewal and pluripotency, and MSCs due to their multipotency and anti-inflammatory properties, have been considered valuable resources in regenerative medicine. The overall aim of this thesis was to investigate the mechanisms by which human ESCs and BM-MSCs enhance wound healing in injured rabbit VFs, and translate this knowledge to a novel cell therapy for patients with severe voice disorders.

Study I
To evaluate the effects of transplanted hESCs in the injured rabbit VFs after 3 months, as well as, to examine whether malignances or teratomas develop from hESCs transplantation in the VFs.

Study II
To establish whether local injection of BM-MSCs in a rabbit model of VF injury can modulate the early inflammatory response and promote the wound healing process.

Study III
To establish whether HA hydrogels offer a safe and efficient means for the delivery of BM-MSCs in a rabbit model of VF injury.

Study IV
The aim of this phase I clinical study was to evaluate the safety of local delivery of autologous BM-MSCs in patients with VF scarring. The secondary aim was to functionally examine the effects of BM-MSC delivery on the restoration of VF function.

3 METHODOLOGICAL APPROACHES

Detailed description of materials and methods used in each study can found in the respective papers (I-IV) included in this thesis. This section provided an overview of materials and methods used in the presented studies.

3.1 MATERIALS

3.1.1 Ethical approvals

Ethical approvals for the use of human ESCs and BM-MSCs were obtained from the regional ethical review board, Stockholm, Sweden (hESC specific S29-06, S115-08, MSC specific ethics DNR 446/00, 2016/1582-3, 2018/1187-32). *In vivo* experimentation on rabbits for these thesis studies were obtained from the Swedish Board of Agriculture (N117/12 and N609/12). The clinical use of autologous BM-MSCs in patients with severe voice hoarseness was approved by the regional ethical review board, Stockholm, Sweden (DNR 2010/1650 and DNR 2014/51432), with manufacturing of the cells conducted in Vecura under good clinical practice according to permissions from the Swedish National Board of Health and Welfare (952/2009, 6.3.3-8874/2011, 6.1.3-9791/2013, 6.1.3-16411/201). All MSC donors were informed and provided written consent as per the Helsinki declaration. The clinical study was registered at ClinicalTrials.gov, ID: NCT01981330.

3.1.2 hESC preparation and characterization

hESC cell line HS181(46;xx) was obtained by the local hESC network at Karolinska Institutet, provided by the Hovatta group(223). Briefly, the blastocyst was obtained from the consented patient and cells from the ICM isolated by removing zona pellucida. The obtained cells from ICM were transferred to culture dishes containing feeder cells (commercially obtained human foreskin fibroblasts), which were mitotically inactivated by 35 Gray irradiation as described(224). The cells were cultured in KnockOut^{TM} Dulbecco's modified Eagle's medium (DMEM) containing glutamine (2nmol/1L), fetal calf serum (FCS; 20% [v/v]), β-mercaptoethanol (0.1mmol/1), non-essential amino acids (1% [v/v]) and recombinant leukemia inhibitory factor (1µl/ml). After 9-19 days of initial growth, cell aggregates were sliced using glass capillaries in mild dispase solution, and aggregates removed from the culture dishes. Three or four undifferentiated hESC colonies were aspirated directly from culture plates in 1ml of saline solution. Cells were, injected in 0.1ml (approximately 10^4 cells) into the LP and/or to the superficial part of thyroarytenoid muscle of each VF. Passage 33 cells were used for study I included in this thesis. The undifferentiated hESCs were characterized by immunohistochemistry and confirmed as positive for Oct-4, TRA-1-60, and SSEA-4.

3.1.3 Isolation of human MSC, expansion and characterization

Human MSC were obtained from iliac crest bone marrow aspirate of healthy donors as previously described(216, 225). Briefly, the aspirate was diluted in 2 volumes of phosphate buffered saline (PBS) and centrifuged, before the mononuclear cells were separated on a Percoll® gradient and resuspended in DMEM-low glucose supplemented with 10% (v/v) FCS, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 0.25µg/ml Fungizone® (studies II

and III) or platelet lysate (final concentration equivalent of 9 x 10^7 platelets/ml; study IV). Cells were plated at a concentration of 1.6 x 10^5 cells/cm^2 and cultured until they reached 80-90% confluency. The adhering MSCs were detached using 0.05% trypsin-EDTA (studies II and III) or TrypLE™ (study IV) and further expanded, with a cell seeding density of 3 x 10^3 cells/cm^2. MSCs were used at passages (P) 3-6 for studies II and III, and P1 for study IV. Differences in culture protocols exist for study IV in order to bring production in line with good clinical practice. Platelet lysate and TrypLE™ replaced FCS and trypsin-EDTA as xeno-free alternatives.

MSCs used in studies II and III had been previously analysed for their capacity to differentiate down the adipogenic and osteogenic lineages(226). Flow cytometry was performed to confirm an MSC surface marker profile, as per ISCT minimal criteria, defined as $CD73^+$, $CD90^+$, $CD105^+$, HLA-I^+ and $CD14^-$, $CD34^-$, $CD45^-$, and HLA-II^- for MSCs used in all studies(57).

3.1.4 Rabbit model of vocal fold injury

New Zealand white rabbits, with a body weight of 3-4 kg, were used for the pre-clinical *in vivo* experiments conducted as part of studies I (n=16), II (n=31) and III (n=18). We have previously developed this rabbit model of VF injury in house, and extensively validated its potential in studying VF injury and the effects of locally administered cell therapy in promoting tissue repair(55, 74, 160, 161, 189, 206, 227). Rabbits represent a good system, possessing large enough VFs to be able to successfully insert a laryngoscope and perform clinically relevant experiments. Furthermore, as discussed in section 1.1, rabbits possess a similar tissue architecture and healing response to that seen in humans, providing a model to access the wound healing cascade after injury within the VF and the potential therapeutic effects of MSC administration.

All animals were carefully monitored before and after the surgical procedure, and experimentation was conducted in line with Karolinska Institutet's internal regulations, as well as the Swedish regulations on animal care ethics conforming to Directive 2010/63/EU. Animals were anesthetized, and the larynx was viewed and examined using a pediatric laryngoscope and rigid endoscope (Figure 8). The injury was made in the superficial thyroarytenoid muscle, mimicking an injury akin to that would be induced during surgical resection of a VF scar within the clinical context.

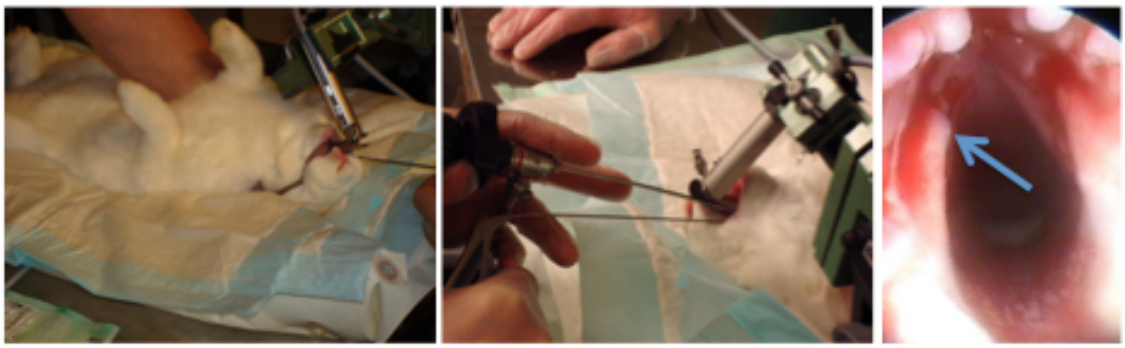

Figure 8: Rabbit vocal folds were visualized using a rigid endoscope, and injuries induced in the superficial thyroarytenoid muscle (arrow). hESCs and MSCs were injected immediately after injury into the wound site.

Animals were sacrificed at 6 and 12 weeks after hESC injections (approximately 10^4 cells/ per VF) in study I. Animals included in study II were sacrificed early time points (days 2 and 4) to evaluate early response by MSCs (0.1-0,15x10^6 cells/per VF) to inflammation within the injured VF, and at later time points (up to day 25) to evaluate tissue architecture. In study III, we further investigated whether injection of the cells in a HA hydrogel carrier (Auxigel^{TM}) may enhance tissue repair and/or retention of the MSCs at the site of injury, and also to establish whether the HA hydrogel could be resorbed, as well as, the long-term effects on tissue repair. For the clinical trial in study IV, the MSCs (0.5-2x10^6 cells in a 0.9% [v/v] saline solution supplemented with 10% AB Rh+ plasma vehicle) were injected into the scarred/damaged region of the LP and thyroarytenoid muscle using a Medtronic Xomed 27G laryngeal injector.

3.1.5 Patient Characteristics

Sixteen patients (11 males and 5 females) were selected for our phase I clinical study (study IV) based on inclusion criteria of severe hoarseness, vocal fatigue and/or VF scarring. Patients were receiving no other treatment and were >18 years at the point of inclusion. Exclusion criteria included no active inflammatory condition of the larynx or laryngeal papilloma, no diagnosed or suspected local malignancy, and female patients should not be pregnant. The patients were aged between 30-74, with a mean age of 54. All patients were negative for human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), human T lymphotrophic virus (HTLV), syphilis and lues. Videostroboscopic examination or a highspeed camera was used to diagnose the patients. All the patients had shown manifest symptoms of strained voice and severe dysphonia, and all had undergone voice therapy treatment for 5-10 sessions by a speech and language pathologist with no improvement observed.

3.2 METHODS

3.2.1 Histological staining techniques

Several histological staining techniques were employed in studies I, II & III to allow visualization of changes in tissue structure and cellular composition, in response to injury and MSC administration, within the VF tissue. Processing of tissue for histology consists of five different stages including fixation, processing, embedding, sectioning and staining(228). The chemicals used for fixation of the tissue preserve the natural tissue structure, as well as, protecting the cell structure from degradation. Various fixative chemicals can be used, but formaldehyde, as used in studies I, II and III, is the most common(229, 230). Paraffin wax used for the embedding process, maintains the integrity of the tissue during sectioning and transfer to slides for staining.

3.2.1.1 Hematoxylin & eosin (H&E) staining

This staining technique provides a simple means to visualize tissue architecture and a wide range of ECM, nuclear and cytoplasmic features. Introduced by Wissowzky in 1876, H & E has been extensively used in both biological and histopathological research(231). The blue-purple color of hematoxylin stains nucleic acids and eosin stains proteins pink, without specificity. In tissue, the nuclei stain blue and cytoplasm, ECM and erythrocytes appear in different shades of red-pink. H & E staining was used in studies I, II and III to verify basic tissue histology after injury of the VFs, the degree of inflammation by viewing lymphocytic

infiltrate and the level of fibrosis. Although the H&E stain is useful in understanding abundant tissue structural information along with morphological changes, limitations do exist. Its non-discriminate staining of ECM components and cells means further analysis is required for detailed investigation of individual cell types and changes in tissue composition.

3.2.1.2 Masson's trichrome staining

Masson's trichrome stain was employed in study II to localize connective tissue proteins, including total collagen fiber deposition and their structure and organization in the VF tissue after injury. This three color staining protocol, that has historically evolved from the original recipe reported by Mallory, was adapted by Masson and Gomori, before further modification, by Lillie, to that used in this thesis(232, 233). Analine blue is used to stain collagen blue, and muscle and cytoplasm color pink with staining using Beibrich scarlet-acid fuschin. Nuclei are stained with Weigert's iron hematoxylin. The use of acetic acid allows color development to appear more delicate and transparent, whilst Bouin's solution intensifies the final coloration for contrast between the stains.

3.2.1.3 Elastin Staining

Staining of elastin composition and organization within the VF was performed using a hematoxylin-iodine-ferric chloride solution, with a Van Gieson counterstain, allowing the visualization of collagen fibers (red), muscle and other matrix molecules (yellow; study II). Elastin fibrils are refractive, and thinner than collagen fibers, usually appearing as branching fibers or sheets in the tissue(234). Ferric chloride and iodine bind to the elastin fibers and act as oxidizers in the conversion of hematoxylin to hematein(235). Since the sections are overstained in this manner, there is a need for differentiation, which is achieved by use of a diluted ferric chloride solution, allowing the tissue-mordant-dye complex to be broken down. One disadvantage of this stain is that differentiation of each slide cannot be achieved using a standardized procedure, rather each section requires monitoring during the process, as the time required depends on the amount of elastin present in the tissue.

3.2.1.4 Alcian blue staining

Alcian blue solution contains copper pthalocyanine and is a polyvalent basic dye that carries a positive charge. It primarily stains acidic polysaccharides, such as GAGs, as well as, sulfated and carboxylated acid mucopolysaccharides and glycoproteins (sialomucins)(235). This method was used to visualize the HA delivery vehicle after injection into the injured VFs in study III.

3.2.2 Parallel plate rheometry

Parallel plate rheometry was used measure the viscoelastic properties of VF tissue. We employed this technique in study I to verify viscoelastic shear properties and evaluate the functional effects of hESC injection on VF vibrations. This method was performed as described previously(30, 227). In this experiment, an AR 2000 rheometer was used, consisting an 8mm diameter stationary lower plate separated by 0.5mm from a rotating upper plate. This produces sinusoidal shear forces with small amplitude oscillations at increasing frequency (0.01-15Hz). The VF tissue samples were placed into the rheometer and measurements performed in the linear region. The measurement of material resistance to shear flow (dynamic viscosity n' Pa.s) and material stiffness (elastic modulus, G' in Pa) was recorded and analyzed.

3.2.3 Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)

This assay was developed to detect localization of apoptotic DNA fragments in tissue by Gavriell *et al*(236). This assay was utilized to detect levels of cell death close to the injury site within the VF and establish whether MSC administration could ameliorate this damage (study II). Briefly, the tissue is incubated with labeling buffer containing biotinylated deoxyuridine triphosphates, which are coupled to the 3' hydroxyl terminus of DNA by terminal deoxynucleotidyl transferase(237). Binding of the labelled nucleotides to the DNA ends is visualized by incubation with streptavidin-conjugated horseradish peroxidase (HRP), and development using 3,3'-Diaminobenzidine (DAB). Methyl green counterstain is cationic and will bind to DNA, exhibiting greater uptake by cells, which are pyknotic, mitotic, or apoptotic(238). Cells with fragmented chromatin, a characteristic of apoptosis, appear dark brown after staining. However, this assay can be limited by the fact that swollen, necrotic cells can also exhibit brown staining within the cytoplasm, as well as, the nucleus. Likewise, eosinic cells can exhibit greater methyl green uptake, therefore the need for appropriate controls and careful interpretation of the data is essential.

3.2.4 Immunohistochemistry

Immunohistochemistry (IHC) and immunofluorescence (IF) are essential methods for a biological researcher to verify the distribution, of a given molecule within tissue sections via antigen-antibody reaction. In study II IHC was employed to detect CD163 expression on the surface of monocytes/macrophages within the injured VF. The sections were stained with a monoclonal, murine antibody raised against human CD163, that had been demonstrated to cross-react with the lapin antigen. A biotinylated goat anti-mouse IgG secondary antibody was used to provide the substrate for binding of avidin, and subsequently HRP. Color development was performed using the chromogenic dye DAB, which when oxidized by HRP forms a brown precipitate that can be observed using bright field microscopy(239). Mayer's hematoxylin was used as a nuclear counterstain as previously described.

In studies I and III we performed IF for detection of type I collagen within the VF tissue using a monoclonal, murine antibody against bovine type I collagen, that cross-reacts with the lapin antigen. A Cy™3 conjugated goat anti-mouse IgG secondary antibody was used in conjunction, allowing visualization of the collagen fibrils. A VECTASHIELD® mountant containing the nuclear counterstain, 4′,6-diamidino-2-phenylindole (DAPI) was added to the stained tissue to prevent bleaching of the fluorescent signal and stain nuclei blue. Relative type I collagen levels were evaluated using in house custom software.

The IHC method is useful in understanding and analyzing positive signals in larger areas of tissue, while IF allows subcellular localization of target molecules. The number of target molecules that can be detected also varies between these methods. IHC can be used for two molecules including counterstaining, whereas IF can be used to visualize multiple target molecules, limited only by the number of fluorophores available(240, 241). However, IHC and DAB based detection provides permanent signals, and stained sections can be stored forever. In contrast, fluorescence signals are bleached if they are exposed to a light source or in long-term storage.

The IHC stained tissue sections from study II were scanned using a 3DHISTECH MIDI Scanner, digital microscope(242). This technology provided high resolution, whole slide imaging, with digital images importable into the associated Pannoramic Viewer software.

The generated images were then compatible with CellProfiler software, allowing quantitative assessment of CD163 levels within the tissue(243, 244).

The major limitation of IHC and IF within these studies was the lack of antibodies suitable for use in rabbit tissue. During the early phases of study II, significant time and effort went into testing and validating appropriate antibodies for visualization of the T cell markers CD3, CD4 and CD8, the macrophage marker CD11b, and immunomodulatory molecules associated with MSC mode of action IL-6, IL-10 and IDO. These studies proved unsuccessful. This is the primary reason why only CD163 was subsequently used in the published manuscript.

3.2.5 Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) is a molecular cytogenetic technique widely used in the field of cytology. This technique, developed in the early 1980s uses the binding of fluorescent DNA probes to complementary nucleic acid sequences allowing their visualization via fluorescence microscopy(245). This technique was employed in studies I, II and III to detect human MSCs at various time points after their local injection into injured rabbit VFs. This exploitation of species differences between ESC, MSCs and VF tissue, allowed us to use FISH as a measure of ESC and MSC persistence in the tissue after administration. This method had been previously established within our lab for this purpose in the rabbit VF injury model(55, 160, 161, 189, 206).

Tissue samples are denatured at 95°C, before treatment with pepsin A, a protease which can digest cytoplasmic proteins. Both of these steps are essential to ensure optimal binding of the DNA probe. For the purposes of these studies we used CEP X (DXZ1) SpectrumGreen Probe to detect ESCs and SpectrumRed™ Y chromosome whole genomic probe to detect MSCs (from a male donor) within the tissue. The probe was denatured to ensure optimal binding to its complementary DNA sequence and hybridized overnight at 38°C. Slides were mounted with VECTASHIELD® containing DAPI counterstain as previously used in section 3.2.4 for IF.

3.2.6 Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qPCR) is a commonly used method in molecular biology to quantity gene expression, which was developed in 1992 for target amplification and detection using either fluorescent dyes or probes. This method allows the quantification of the exact amount (relative or absolute) of amplified gene expression(246). Total RNA was extracted from rabbit VF tissue (studies II and III) or human MSCs (study III). Tissue from the animal studies was preserved in RNAlater® solution prior to processing, ensuring stability of the RNA within the samples and inactivating RNase activity. Total RNA was extracted from tissue using the Ribopure™ RNA Isolation kit. This kit contains TRI Reagent® to homogenize the tissue, prior to treatment with bromochloropropane, a less toxic equivalent to chloroform, which aids in the separation of the nucleic acids. For isolation of total RNA from MSCs, cells were lysed in RLT buffer supplemented with ß-mercaptoethanol (to degrade RNases), using the RNeasy® mini kit. Both the Ribopure™ and RNeasy® kits use a spin column-based method for separation of RNA, binds the nucleic acids to the filter membrane, allowing washing with ethanol and finally wash solution, to remove any contaminating ethanol, which would inhibit downstream enzyme activation. RNA is eluted

in molecular grade water, before quantification and assessment of quality using a NanoDrop spectrophotometer.

cDNA template generation from eluted RNA was performed using the RT^2 first strand kit (VF tissue) or high capacity cDNA reverse transcriptase kit (MSCs). In study II, gene expression studies were conducted using standard qPCR methodology, whereas in study III this method was used in combination with an RT^2 profiler system consisting of a panel of 84 rabbit inflammatory cytokines and receptors. This array was used to generate preliminary data as to the key inflammatory cytokines upregulated in response to VF injury within the rabbit. Analysis of data from 2 injured rabbit VFs and 1 uninjured, control VF, provided us with a battery of cytokines, which provided information as to the key cytokines the injected MSCs may be exposed to and therefore modulate. These included IL-1β, IL-8 and CCL4 which were further investigated at mRNA and protein levels in studies II and III.

Standard qPCR was performed using human MSC cDNA gene specific primers generated using Roche's universal probe library. In contrast such extensive databases for the rabbit genome are not available and therefore gene targets investigated using qPCR in the rabbit tissue was limited by those commercially available. For both human and rabbit target genes, the house-keeping gene β-actin was used for normalization of expression levels.

3.2.7 Preparation of hyaluronan hydrogel

AuxiGel™, used in these studies, combined an aldehyde derivative of HA with poly-vinyl alcohol (PVA) to generate a chemically modified HA hydrogel in studies III and IV of this thesis. Both HA and PVA components are mixed with PBS at concentrations of 15mg/ml and 2.5mg/ml respectively. HA and PVA are loaded into 2 1ml sterile luer-lock syringes at a ratio of 3:1, previously optimized to ensure that stiffness of the gel generated would be suitable for combination with the MSC suspension, and injectable through a 27G needle upon delivery. Both syringes were connected using a luer-lock adapter and the components mixed at room temperature for 5-10 cycles, 1 cycle determined as movement of all the material from 1 syringe to the other with duration time of 5 to 20s (for optimal mixing, while minimizing air bubble formation) for each cycle.

For experiments in study III, the HA hydrogel was mixed with MSC cell suspension in cell culture media (*in vitro* experiments) or PBS (*in vivo* studies) before injecting through a 27G needle. In study IV, where MSC: HA hydrogel mix was injected into the patient's VF tissue, MSCs were suspended in 0.9% (v/v) saline supplemented with 10% (v/v) human AB Rh+ plasma.

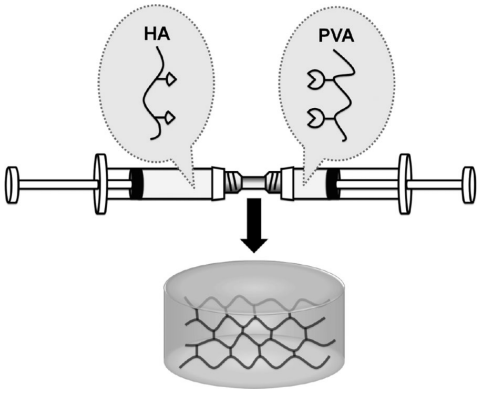

Figure 9: Schematic illustration of Auxigel™ preparation. Hyaluronan (HA) and poly-vinyl alcohol (PVA) components are mixed in connecting syringes, generating a chemically cross-linked hydrogel. The figure is reproduced with permission from *Piskounova et al., Macromolecular Material and Engineering, 2011, 296,944-951.*

3.2.8 Determining the effect of HA on MSC number

We used the CyQUANT® Cell Proliferation assay to assess the effect of HA gel incorporation on MSC viability and proliferation *in vitro*. The key component of this assay is the CyQUANT® GR dye that displays a large fluorescence enhancement when it binds nucleic acids. MSCs that were incorporated in the HA gel were snap-frozen at days 0, 1 and 3, and subsequently lysed in buffer containing the CyQUANT® GR dye, providing access to the nucleic acids(247). This system is sensitive, exhibiting a linear detection range, using fluorimetry, between 50-50,000 cells and is not dependent on cellular metabolic activity like assays such as ^{3}H-thymidine uptake. The primary advantage of this sensitivity is that relative increases and decreases in cell number can be evaluated compared to the day 0 reading. This allowed us to use this assay for both the purpose of investigating cell proliferation and survival within the HA gel in study III.

3.2.9 Establishing the effect of HA on MSC migration and invasion in response to pro-inflammatory signals

To verify whether HA hydrogel incorporation modulated cell migration, chemotaxis and invasion in response to pro-inflammatory factors, the Cultrex 96 well Collagen I Cell Invasion assay was employed in study III. This high-throughput, 96 well format, assay uses a Boyden chamber approach to evaluating cell migration through an 8μm polyethylene terephthalate membrane. In order to evaluate migration, MSCs +/- HA hydrogel incorporation were applied to the top chamber and allowed to migrate through the uncoated membrane. The addition of our selected pro-inflammatory factors upregulated during early VF injury response, IL-1β, IL-8 and CCL4, were added to the bottom chamber to evaluate directed migration of the MSCs. To also evaluate invasion potential of the MSCs, the chamber membrane was precoated with type I collagen, providing a matrix, as would be seen in the VF tissue, which the MSCs would need to digest and remodel in order to migrate through to the underside of the chamber. After 24 hours incubation at 37^{o}C, the migrated cells were dissociated from the membrane and stained with calcein-AM. This dye freely passes through the cell membrane and is cleaved by intracellular esterases to calcein, which fluoresces brightly and allows cell number to be quantified by fluorimetry.

3.2.10 Enzyme linked immunosorbent assay

In study III, sandwich enzyme linked immunosorbent assays (ELISA) were used to evaluate secreted levels of IL-6, IL-8, HGF, VEGF and TGFβ1 by MSCs +/- HA hydrogel incorporation in response to IL-1β, IL-8 and CCL4 exposure. Monoclonal antibodies targeted against the human soluble factor of interest were coated on to high-binding ELISA 96 well plates overnight at room temperature. Residual binding sites were blocked using a bovine serum albumin buffer. Conditioned media from MSCs exposed to the above conditions, or a standard curve of the purified protein of interest (concentration range for linear detection optimized by the manufacturer) is then incubated in the plate for 2 hrs to establish binding of the ligand to the antibody. The plates are, after washing, incubated with a biotin tagged secondary antibody raised against the human protein of interest. Binding of the secondary antibody can be detected through incubation with HRP conjugated streptavidin, before development of the colour using a 1:1 mix of hydrogen peroxide and tetramethylbenzidine. This reaction is stopped with the addition of sodium hydroxide, which generates a stable yellow product. The intensity of this product linearly correlates with the concentration of the protein of interest in your test sample.

Evaluation of TGFβ1 using these assays requires prior preparation of your sample prior to incubation with the primary antibody. TGFβ1 can exist in both latent (not immunoreactive) and bioactive forms and therefore in order to measure total TGFβ1 levels, samples are pretreated with hydrochloric acid to remove the latent peptide, rendering a detectable form of the growth factor. Serum containing media, in which the MSCs are grown, contains a considerable amount of latent TGFβ1, as well as, other growth factors. It is therefore important than unconditioned media controls, at each dilution that the samples will be tested, is also added to the plate in order to give an accurate background control. As there is batch to batch variability in the contents of serum, this background control is matched to each experiment run.

3.2.11 Clinical evaluation of vocal fold function

The patients included in the clinical study (study IV) were selected with manifestation of symptoms of severe voice problems or dysphonia. Both methods of clinical assessment and self-reporting were utilized in this study. Initially we performed high-speed examination and videostroboscopy to examine the VF vibration and function. These methods allow the identification of various parameters of VF function such as VF edges, closure, vibratory characteristics (vibration symmetry without irregularity or stiffness), and structural characteristics (edema or masses that interfere with phonation)(248). The high-speed videostroboscopy method allowed visualization of glottal dysphonia, and also recording of VF motions with enhanced temporal resolution(249). In our studies, we used a HiSpec 1 camera for high-speed recordings of VF vibration, which were digitalized for further analysis using High-Speed Studio (HSS) software. VF glottal closure and amplitude during vibration was digitized, normalised to membranous VF length and measured with the HSS software. The open/closed coefficient during vibrations was calculated using HSS from kymograms. VF parameters such as glottal closure, amplitude of vibration and mucosal waves were analyzed by three selected phoniatricians, who were blinded regarding the patients' clinical history. Video recordings were rated in a pairwise manner from pre-operative recordings and at 12 months after surgery.

PTP was recorded as a measurement of VF elasticity and vocal onset effort(4). This was measured from the minimal intra-oral pressure using a pressure transducer and a 4mm plastic catheter placed in the corner of the patient's mouth during repeated "pa" syllables at habitual

pitch. The Voice Handicap Index (VHI) scale was used to rate a patient's subjective voice symptoms consisting of physical, functional, emotional aspects of daily living. This standardized scale consists of 30 questions or statements validated for the Swedish language, for the patients to answer(250).

4 RESULTS AND DISCUSSION

4.1 STUDY I

Tissue injury or scarring in the VF disrupts viscoelastic properties in the tissue and leads to stiffness in the LP, causing severe voice problems(3, 4). hESCs have been considered as a potential resource for cell therapy in regenerative medicine. In our previous study, hESCs injected into injured rabbit VFs demonstrated human tissue generation (cartilage, epithelium, glands and muscle) close to the injection site in the VF tissue(55), improving viscoelasticity of tissue and VF function.

4.1.1 The role of ESCs in tissue formation and wound healing

ESCs have been shown to aid tissue repair within several animal models(251). For example, ESCs have been demonstrated to promote cutaneous repair in nude mice through differentiation into basal keratinocytes(252, 253). Likewise, in disease models, ESCs have been suggested to improve symptoms of Parkinson's disease via neuronal differentiation(254-256). Furthermore, their differentiation into hepatocytes and chondrocytes(257-260), pancreatic tissue(261, 262) and blood cells(263, 264) have also been reported. Due to their capacity to self-renew and pluripotency, ESCs may have been considered as a potential resource in regenerative medicine(265). Based on knowledge from the literature, and the results obtained from our previous study on the histology and properties of VFs one month after ESC injection(55), we now designed a study to see the long term (3 months) effects of hESCs in the injured VF tissue. The findings from this study are described below.

4.1.2 Key findings

1) hESC administration into injured rabbit VFs increased viscoelastic properties to those comparable to normal VFs.
2) Histology was improved and indicated normal VF architecture after hESC treatment.
3) IHC results depicted reduced collagen deposition in the injured VFs treated with hESC compared to untreated VFs.
4) LP thickness was reduced significantly in hESC treated VFs compared to untreated VFs. The difference in the thickness of the LP between hESC treated VFs and normal VFs was not significant.
5) hESC derivatives such as teratoma and malignances were not found in the treated VFs at 3 months.
6) Transplanted hESCs were detected after six weeks in the injured VFs.

4.1.3 Discussion

Our first study indicated that local injection of hESCs improved VF vibration after one month(55). The findings of this 3 month study confirm the positive effects of hESC injection and indicate that the effect is sustained over time. The results from H&E staining indicated

that the VF tissue organization was improved with hESC treatment, consistant with reduced LP thickness. Additionally, type I collagen levels were significantly reduced in the hESC treated VFs compared to untreated, injury only controls. The injected hESCs stayed close to the injection site within the LP and muscle up to six weeks after transplantation. No teratomas and malignances were evident post-hESC injection after three months in the treated VFs.

Although our findings indicate beneficial effects of injecting hESCs into the injured VFs in both our 1 and 3 month studies, there are several other obstacles that remain to be resolved, regarding these cells, before moving onto further basic research or into clinical application. One limitation for our research is that hESCs are not known be immune suppressive in the presence of inflammation. As our studies deal mainly with inflamed or scarred VF tissue, we decided to switch to using BM-MSCs as a potentially better cell source to conduct further studies in our research discipline.

4.2 STUDY II

Damage and scar formation within the VFs leads to significant changes in tissue viscoelasticity and shear strength, disturbing the normal mucosal wave during speech or phonation resulting in severe voice problems(5). Previous studies, including those from our group, have demonstrated that local administration of MSCs can reduce scar formation in the injured and scarred VF(160, 161, 183, 189, 212). Limited information regarding the MSC mode of action in promoting these healing effects has been delineated, therefore in this study we investigated the effect of MSC injection on the early inflammatory phase of wound healing after VF injury.

4.2.1 MSC modulation of acute injury response

MSCs have been shown in multiple organ systems to play a pivotal role in tissue homeostasis and repair(219-222, 266-269).Their ability to home to sites of injury and modulate the activity of both immune cells and resident stromal fibroblasts has established fundamental reparative roles for MSCs in all phases of the healing cascade(142, 270, 271).

No effective treatment for chronic scarring within the VF exists currently, but great promise has been placed on the development of novel cellular therapeutics, including the use of MSCs. The aim of this study was to ascertain how local administration of MSCs during the acute inflammatory phase, as would be induced upon surgical resection of VF scar tissue, could modulate the healing response.

4.2.2 Key findings

1) MSC administration expedited the resolution of tissue inflammation as demonstrated by decreased lymphocyte infiltrate within the VF tissue of treated animals compared to injury only controls.
2) These histological observations were further supported by a decreased mRNA expression, in the VF tissue, of the pro-inflammatory cyto/chemokines IL-1β and IL-8 at day 2 compared to injury only control animals.
3) Decreased mRNA expression of the monocyte recruitment and activation factors M-CSF, monocyte chemoattractant protein -1 (MCP-1) and CCL1 within the MSC treated

VF tissue compared to controls, further indicated that the wound healing response was accelerated by the presence of MSCs, promoting inflammation resolution and transition to the proliferative phase of the wound healing response.

4) Increased levels of the anti-inflammatory macrophage marker, CD163, was observed in the MSC treated VFs compared to controls, supporting an immunomodulatory role by the MSCs.
5) MSCs appeared to reduce the level of cell death at the site of injury compared to injury only controls.
6) Inflammation resolution coupled to improved repair tissue composition, with the collagen architecture organized parallel to the tissue surface, akin to that seen in healthy VF tissue.
7) Despite multiple therapeutic effects on tissue repair in the VF, low level persistance of the administered MSCs was seen.

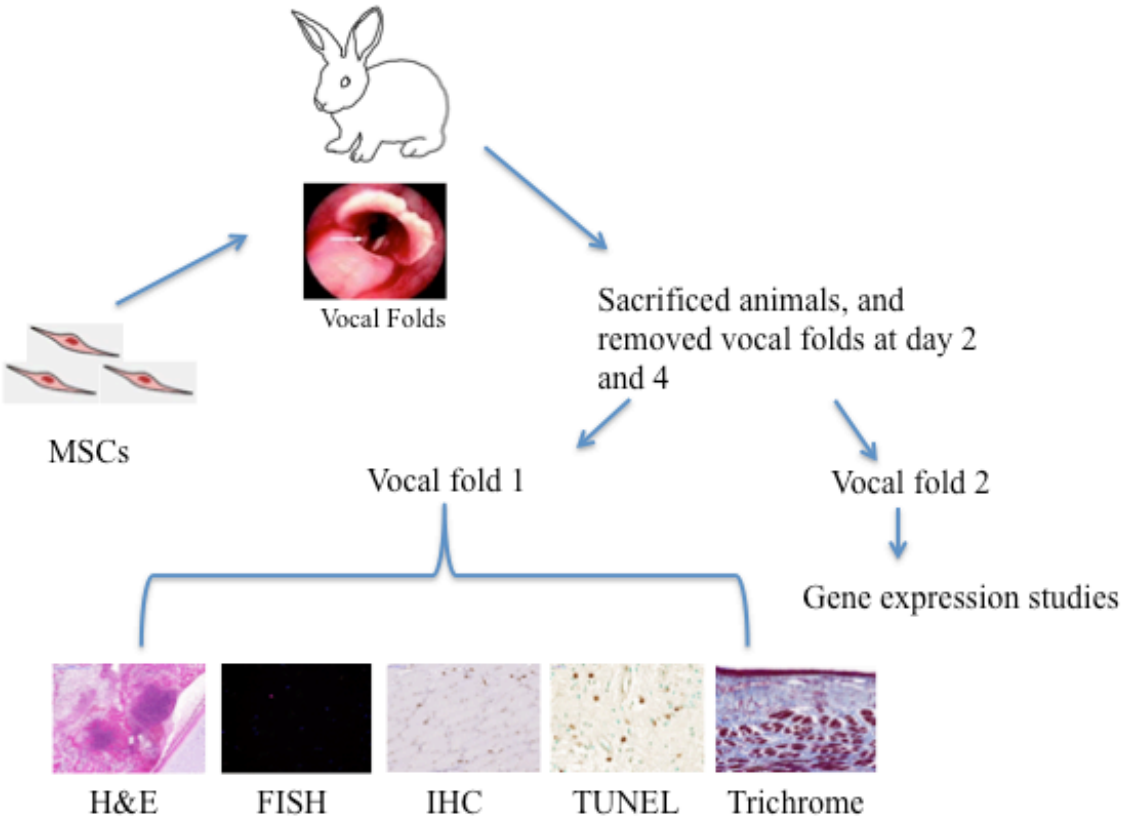

Figure 10: Summary flow diagram illustrating the study design employed in study II. Abbreviations: FISH – fluorescence *in situ* hybridization; H&E – hematoxylin and eosin; IHC – immunohistochemistry; MSCs – mesenchymal stromal cells; TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labeling. All composite images are used with permission. MSCs: *Wu W et al., Molecular and Cellular Proteomics. 2018 Aug;17(8):1502-1514(272)*

4.2.3 Discussion

This study used our previously optimized rabbit model to induce an acute injury and evaluate the effect of local administration of MSCs at the time of injury in enhancing the healing process.

Histology, in conjunction with qPCR analyses, confirmed a large lymphocytic infiltration post-injury, which was quickly resolved with the administration of MSCs. Furthermore, significant lowering of levels of pro-inflammatory cytokines, including IL-1β and IL-8 was seen at the mRNA level. This enhanced resolution of the inflammatory phase of the wound healing process could be attributed to both a decreased level of cell death in response to injury and promotion of an anti-inflammatory milieu that encompasses a skewing of M1 pro-inflammatory macrophages and infiltrating monocytes to an anti-inflammatory CD163+ M2 phenotype. This change in inflammatory milieu also impacted on the production and organization of the ECM. Collagen organization was markedly improved in the MSC treated VFs, appearing densely packed and parallel to the tissue surface. These observations correlated well to the improved functionality associated with MSC treated VFs that we and others have previously reported(160, 161, 183).

In line with our previous observations, only low-level persistence of MSCs was evident 2 days post-injection. These findings support the notion that MSCs do not engraft into the VF tissue but are able to promote healing via indirect mechanisms through the local immune and stromal environment.

4.3 STUDY III

Local administration of MSCs into VF tissue has demonstrated enhancement of tissue regeneration and restoration of VF functions(160, 161, 189). Limitations exist however, in the delivery of MSCs within a liquid suspension. These include risk of cell suspension leakage, and an inability to effectively treat large and critical size defects. The aim of this study was to evaluate whether MSCs delivered in HA hydrogel would offer a safe and effective alternative to liquid delivery, holding the cells at the site of injury site and further supporting tissue repair, with the hydrogel acting as a scaffold for larger defects.

4.3.1 HA for the local delivery of MSCs

HA is a natural component of the VF ECM, contributing to its biomechanical properties, as well as acting as a key regulator of cellular interactions and tissue repair processes(26, 27). In its hydrogel form, HA has been successfully used as a delivery vehicle for cells and bioactive factors, as well as a tissue repair agent in its own right(273, 274). This GAG is a non-immunogenic, biocompatible material that is actively resorbed by the tissue, making it an ideal agent for clinical usage, where for over a decade, it has been used in the treatment of glottal insufficiency(275).

4.3.2 Key findings

1) HA hydrogel incorporation of MSCs does not negatively impact their viability or function, but does delay migration compared to liquid suspension cells.
2) MSCs modulate their mRNA expression of matrix remodeling genes from HAS, HYAL and MMP families in response to HA gel incorporation and exposure to pro-inflammatory cyto/chemokines.
3) MSCs contained within HA hydrogels remain responsive to their extracellular environment, upregulating the secretion of immunomodulatory factors, such as IL-6, in response to pro-inflammatory cyto/chemokines.
4) MSCs delivered in HA hydrogel, in a rabbit model of VF injury, demonstrated less cell suspension leakage compared to liquid suspension controls.
5) Hydrogel delivery of MSCs did not induce edema and was resorbed within the 1 month follow-up time in rabbits.
6) MSCs reduced inflammation and promoted repair of the VFs, with no significant differences noted between liquid and HA delivery vehicles.
7) Delivery of the MSCs in HA hydrogel did not improve persistance of the cells within the VF tissue.

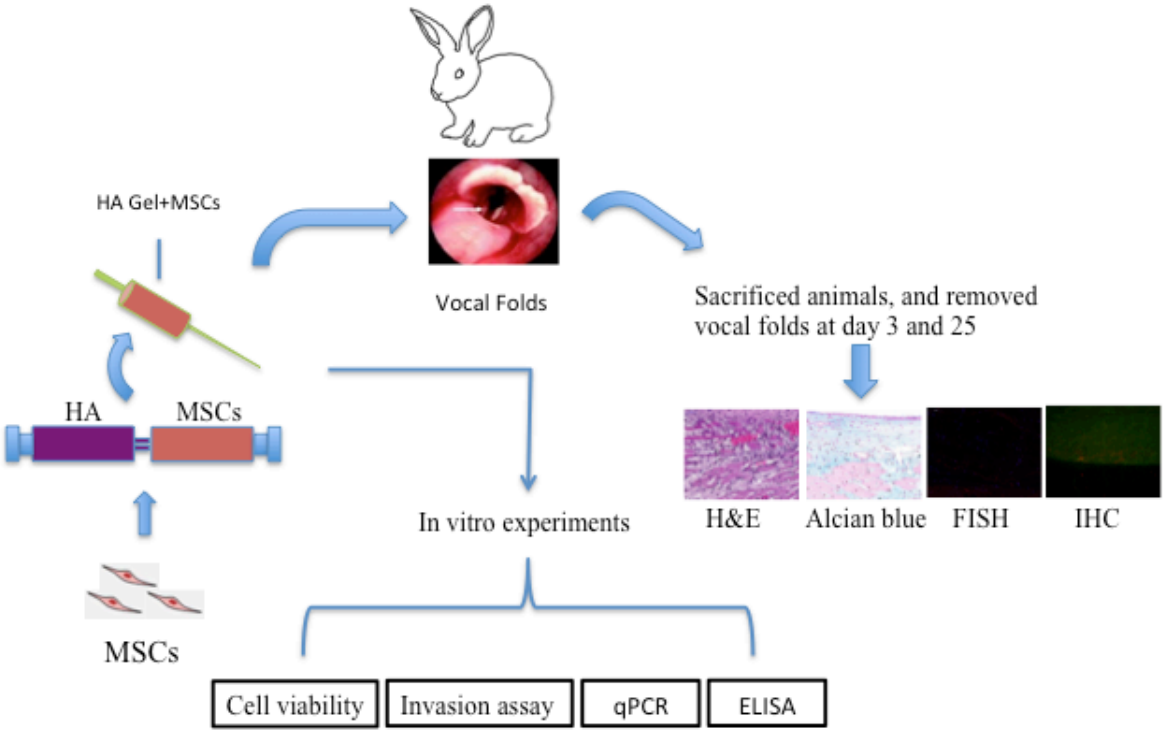

Figure 11: Summary flow diagram illustrating the study design employed in study III. Abbreviations: FISH – fluorescence *in situ* hybridization; HA – hyaluronan; H & E – hematoxylin and eosin; IHC – immunohistochemistry; MSCs – mesenchymal stromal cells; qPCR – quantitative polymerase chain reaction. All composite images are used with permission. MSCs: *Wu W et al., Molecular and Cellular Proteomics. 2018 Aug;17(8):1502-1514(272)*

4.3.3 Discussion

The principle aim of this study was to evaluate whether delivery of MSCs within a HA hydrogel offered a safe alternative to current liquid delivery vehicles, and address limitations in current delivery methods through holding the MSCs at the site of injury, potentially increasing their longevity, minimizing leakage and providing a scaffold akin to the ECM.

Our animal study demonstrated that HA hydrogel delivery of MSCs into VF defects was safe, with no serious adverse side effects, such as edema or inflammation within the VF, in response to the gel. We furthermore conducted a number of *in vitro* analyses to confirm that the MSCs remained viable within the gel and exhibited a delayed, although not halted, migration compared to MSCs in liquid suspension. The presence of a collagen matrix, as seen in the invasion assay setup, did enhance migration out of the HA gel, potentially explaining why there was no increased persistence of the MSCs within the VF tissue with HA delivery. MSCs remained responsive to extracellular pro-inflammatory cytokines relevant to the acute injury milieu that would be seen in the rabbit VF, upregulating the secretion of immunomodulatory factors, such as IL-6 and modulating their expression of key matrix remodeling genes. This was corroborated by the *in vivo* analyses demonstrating consistently reduced inflammation in the presence of the MSCs, irrespective of the delivery vehicle.

We concluded that HA hydrogels can be safely employed for the delivery of MSCs into injured VFs, minimizing leakage of cell suspension during injection and providing a scaffold supporting the healing of critical sized defects.

4.4 STUDY IV

Despite numerous pre-clinical approaches to treating VF scarring, no effective clinical option exists to date. Local implantation of fillers such as fat and HA demonstrate some therapeutic effect, softening the scarred tissue, as well as, injection of growth factors such as HGF and bFGF(208, 276-278). These effects are short lived however, not addressing the underlying pathophysiology of the condition. The aim of this study was to take the knowledge we had learnt from our pre-clinical work, and conduct a phase I/II clinical trial to evaluate the safety of administering autologous human BM-MSCs to patients suffering with severe voice problems.

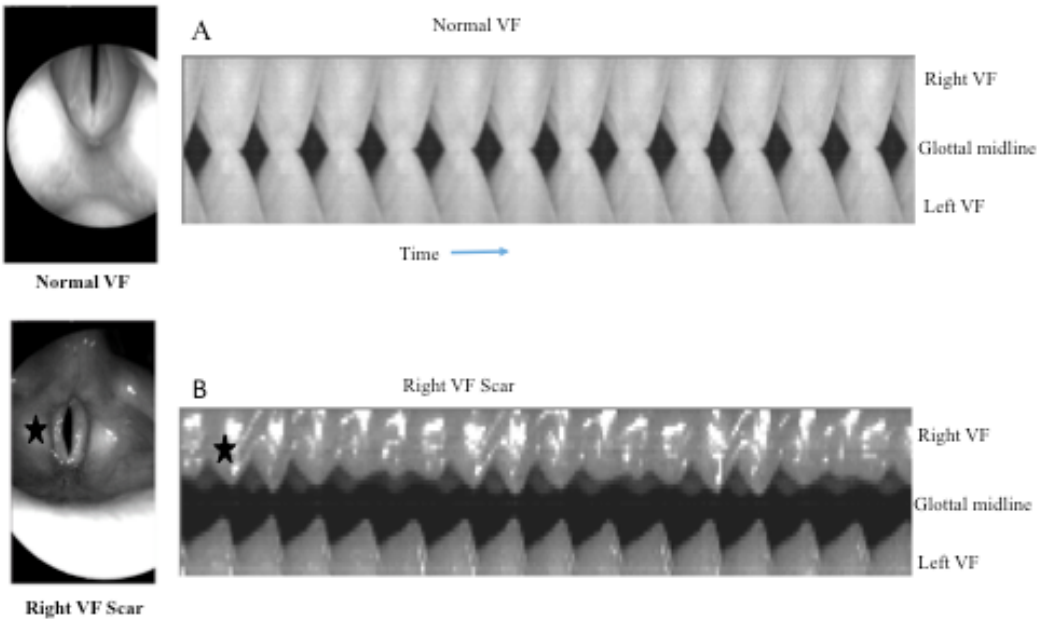

Figure 12: High-speed video kymogram of vocalfold (VF) vibration and glottal closure at the midline, demonstrating that scarring inhibits both VF function and glottal closure. Top picture (A) shows vibrations from normal VFs and lower picture (B) shows reduced vibrations of a scarred right VF (star) with incomplete glottal closure.

4.4.1 MSCs as a clinical treatment for VF scarring

In addition to the trial reported in this thesis, only 1 clinical trial is registered on clinicaltrials.gov (ID: NCT02622464) for the use of stromal cells (autologous adipose-derived stromal vascular fraction) in the treatment of VF scarring. This phase I study, completed in 2018, has yet to report its findings. A case report originating from the same research group has however reported positive findings in terms of safety and improvement in voice function for the female patient(218).

Differences in MSC products, both in terms of cell source and manufacturing protocol, means there is an absolute need to determine safety and efficacy of individual cell products. In this study we used established protocols for the production of a clinical-grade autologous BM-MSC product. With a primary endpoint of safety, we established that our BM-MSC product

was tolerated by patients who received a single dose of locally administered cells into VFs that had been resected of scar tissue in the same surgical procedure. Efficacy served as a secondary endpoint, with both clinical functional assays performed post-procedure, as well as, self-reporting of vocal function.

4.4.2 Key findings

1) In total 16 patients (11 males and 5 females, aged between 30-74) were included in the study with manifestation of severe dysphonia and strained voice.
2) No adverse events were reported in response to MSC administration.
3) Functional evaluation using high-speed laryngoscopy and PTP demonstrated improvements in VF function in 62-75% of the patients (dependent on parameter tested).
4) VHI self-reports indicated improvements in vocal function in 8 of the patients, with the remaining patients reporting no significant change.

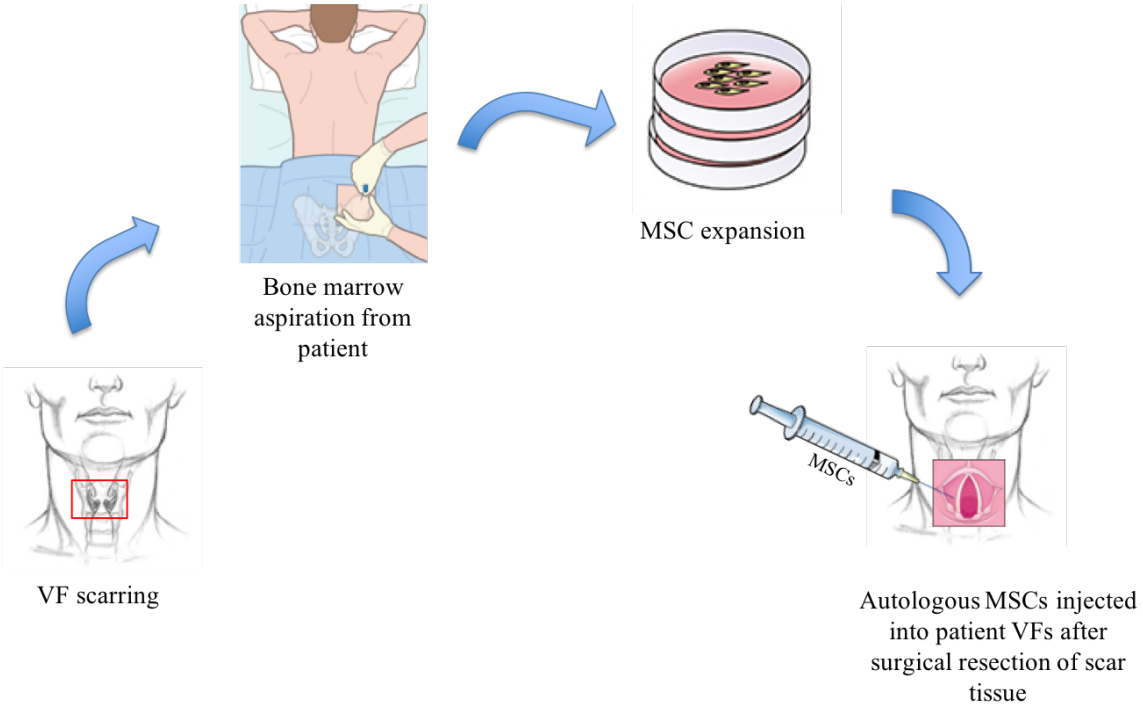

Figure 13: Summary flow diagram illustrating the study design employed in study IV. Patients recruited to the phase I/II trial with damage and scarring to one or both of the vocal folds (VF). Autologous mesenchymal stromal cells (MSCs) were isolated from bone marrow harvested from the iliac crest, and expanded in monolayer to passage 1. MSCs were administered back to the patient at the time of surgical scar resection via local injection into the injured tissue. All composite images are used with permission. Human vocal folds: *Zhang LT, Journal of Biomedical Science and Engineering, 2014 Feb;7(3):130-145(279).* MSC expansion*: Jakubikova J et al., Oncotarget, 2016 Nov 22;7(47):77326-77341(280)*. Bone marrow aspiration*:* www.teresewinslow.com. Syringe and diagram of VFs: smart server medical art (https://smart.servier.com).

4.4.3 Discussion

This is the first phase I/II clinical study documenting use of autologous BM-MSC treatment in humans with VF scarring. Bringing together the knowledge gleamed in studies I and II, here we report clinical translation of our findings for the local administration of autologous BM-MSCs after surgical resection of VF scar tissue.

No acute or long-term side effects were reported from MSC treatment in the 1 year follow-up period post-injection. The most significant finding was in VF vibration capacity/elasticity, where VF vibration parameters were improved for 62-75% of the patients. Self-reporting, using the VHI, suggested that the majority of patients experienced improvement in phonation, being able to speak with less effort.

For most of the patients, clinical improvements became evident after 3 months, indicating a long-term effect from the MSC treatment on healing, supporting our pre-clinical *in vivo* data. Less significant improvements were evident with larger defects, suggesting the potential need for further scaffold development to be used in conjunction with MSC administration. Further investigation of efficacy in a larger trial is warranted for validation of our reported findings.

5 SUMMARY OF MSC STUDIES

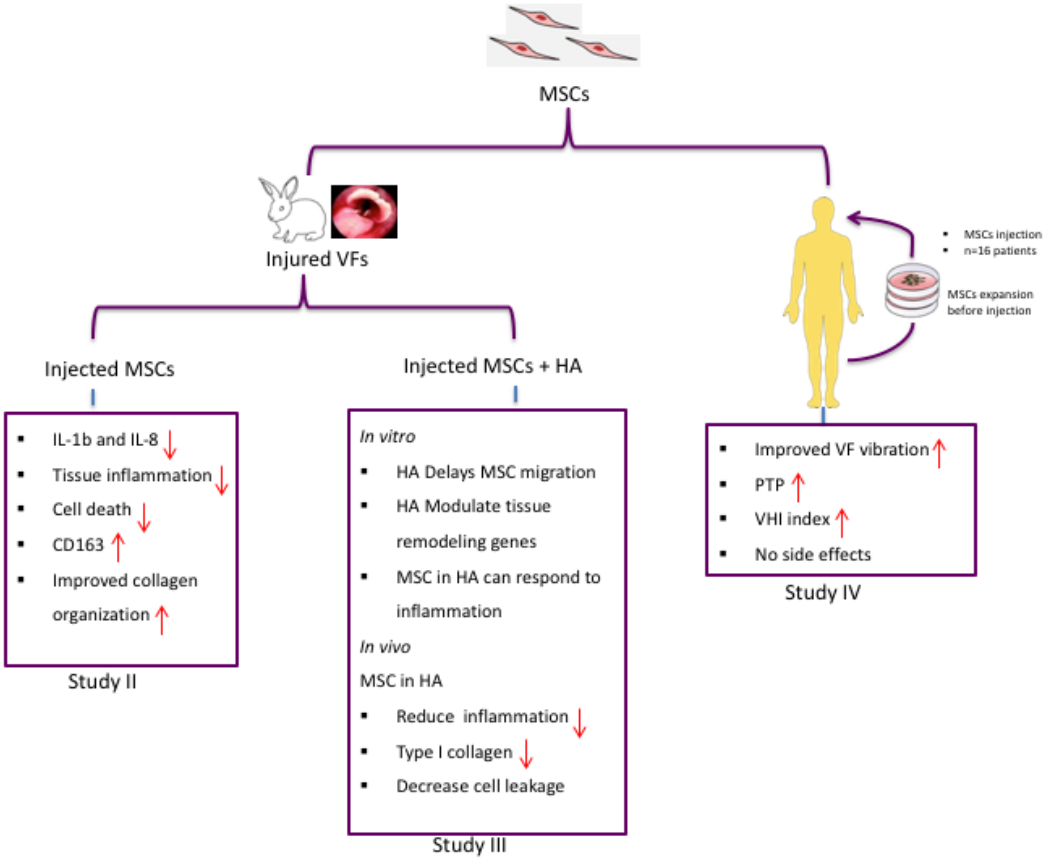

Figure 14: Schematic summary of studies II, III and IV within this thesis. We demonstrate that local administration of mesenchymal stromal cells (MSCs) expedites and improves the quality of healing response within the injured vocal fold (VF), via the dampening of inflammation and cell death, while skewing the extracellular environment to an anti-inflammatory milieu. MSCs are able to do this whether delivered in liquid suspension or a hyaluronan (HA) hydrogel, with the latter option decreasing cell leakage and offering a practical delivery vehicle alternative to the former. Our findings were translated to a phase I/II clinical trial using autologous MSC injection into surgically resected VF scar tissue, demonstrating that this approach offers a safe and novel alternative for the treatment of patients with severe voice problems due to VF damage. Abbreviations: IL – interleukin; PTP - phonation pressure threshold; VHI – voice handicap index. All composite images are used with permission from: human schematic diagram form smart server medical art (https://smart.servier.com). MSCs: *Wu W et al., Molecular and Cellular Proteomics. 2018 Aug;17(8):1502-1514(272).* MSC expansion*: Jakubikova J et al., Oncotarget, 2016 Nov 22;7(47):77326-77341(280)*.

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The development and use of cellular therapeutics, including MSCs, has grown exponentially over the last decade. The low immunogenicity of these cells, thanks to their potent immunomodulatory properties, has made them a popular choice for novel therapy development. MSCs can be isolated from a wide range of tissues, each with their own unique properties pertaining to their *in vivo* role. Much of the knowledge that we can gleam from the wider literature pertains to BM-MSCs, with this particular cell source most well characterized for their role in tissue repair responses and immunomodulation.

VF scarring, due to trauma or other indications, represents significant morbidity for the patient, as well as, a huge societal burden related to healthcare costs and unemployment. For these patients there is no current effective treatment for this condition. Cell therapy, using MSCs, offers a novel approach to this, taking advantage of their ability to reduce inflammation when used in conjunction with surgical resection of scar tissue, and skewing of the endogenous VF cells to promote a functional tissue repair response.

The studies conducted in this thesis have investigated ESC and MSC mode of action from basic *in vitro* investigations, through to an animal model of VF injury, and finally translation into the clinic in the form of a phase I/II trial. Collectively these studies demonstrate the potential for BM-MSC therapy in patients with severe VF damage to improve tissue functionality, and more importantly for the patient, improve phonation. Looking forward, these initial studies need to be clinically validated in a larger cohort. Safety, our primary endpoint, in this clinical trial was met, but there is a need to understand the efficacy of this therapy and how the clinical protocol can be advanced in terms of both an increase in the numbers of patients responding to treatment, and to address whether this approach would be suitable for other related conditions. Furthermore, we have highlighted the limitation of MSC administration for large, critical size defects in the VF. Studies remain to be conducted to understand how this issue can be addressed, including the value of scaffold-MSC or MSC-growth factor/drug combinations. As technology and cell therapies develop, it is hoped that MSCs for the treatment of VF scarring will become an accepted standard line of care.

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