Dissertation

Establishment of a novel in-vitro model for vocal fold scarring

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of "Good Scientific Practice".

Graz, May 2016

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Abbreviations and Definitions

α-SMA	Alpha smooth muscle actin
bFGF	Basic fibroblast growth factor
DAPI	4′,6-diamidino-2-phenylindoldilactate
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assays
EVE	Excluded volume effect
FBS	Fetal bovine serum
Fc	Ficoll
HA	Hyaluronic acid
HAS	Hyaluronic acid synthase
HGF	Hepatocyte growth factor
hVFF	Human vocal fold fibroblasts
ICC	Immunocytochemistry
MMC	Macromolecular crowding
PBS	Phosphate-buffered saline
PCP	Procollagen C-proteinase
P/S	Penicillin/Streptomycin
pVFF	Primary vocal fold fibroblast
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SDS-Page	Sodium dodecylsulphate-polyacrylamide gel electrophoresis
TGFβ1	Transforming growth factor beta 1
VF	Vocal fold
VFF	Vocal fold fibroblast
VHI	Voice handicap index

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Abstract in English

INTRODUCTION: Scarring of the vocal folds (VF) remains one of the main challenges in modern laryngology. Development of novel therapeutic strategies is hampered by the complexity of trials in animal models and the lack of an appropriate in-vitro model. So the aim of this work was to establish an in-vitro fibrogenesis model for vocal fold scarring to enable proper testing of potential therapeutic agents.

MATERIALS & METHODS: Inert macromolecules were used to generate “crowded” conditions and enhance deposition of extracellular matrix in-vitro. Moreover transforming growth factor beta 1 (TGF-β1) was administered in cell culture to ensure fibroblast to myofibroblast differentiation. Hepatocyte growth factor (HGF) and Botox were tested for antifibrotic properties within this model. Trials were conducted using primary vocal fold fibroblasts of rats and an immortalized human vocal fold fibroblast cell line. Experiments were analysed for the biosynthesis of collagen, fibronectin, as well as α-smooth muscle actin (α-SMA).

RESULTS: „Crowding” markedly promoted the in-vitro synthesis of extracellular matrix components like collagen and fibronectin. Related effects could be seen for TGF-β1, also inducing the expression of α-SMA. Combining both treatments potentiated the findings. The antifibrotic properties of HGF could be confirmed in our model.

CONCLUSION: The described model could be of great value by accelerating research in the field of vocal fold scarring, allowing for rapid and standardized testing of potential therapeutic approaches, moreover reducing the number of animal experiments.

Abstract in German

EINLEITUNG: Vernarbungen im Bereich der Stimmlippen stellen eine der größten Herausforderungen in der modernen Laryngologie dar. Die Entwicklung neuer therapeutischer Ansätze gestaltet sich schwierig aufgrund der Komplexität bei der Durchführung von Tierversuchen und ein entsprechendes Modell in der Zellkultur existiert bisher nicht. Das Ziel dieser Arbeit war es ein in-vitro Modell für Vernarbungen im Bereich der Stimmlippen zu etablieren und potentiell antifibrotische Substanzen damit zu testen.

MATERIAL & METHODEN: Inerte Makromoleküle wurden verwendet, um die Bildung einer repräsentativen Extrazellulärmatrix in-vitro zu ermöglichen. Weiters wurde mit Hilfe von TGF-β1 die Differenzierung von Fibroblasten zu Narbenfibroblasten, sogenannten Myofibroblasten sichergestellt. Die Versuche wurden mit primären Fibroblasten aus Stimmlippen von Ratten, sowie einer immortalisierten Zelllinie aus menschlichen Stimmlippenfibroblasten durchgeführt. Analysiert wurde hinsichtlich der Synthese von Kollagen, Fibronektin und α-SMA.

ERGEBNISSE: Die Zugabe von Makromolekülen führte zu einem deutlichen Anstieg der Synthese von Bestandteilen der Extrazellulärmatrix wie Kollagen und Fibronektin. Ähnliche Resultate fanden sich für die Zugabe von TGF-β1, was vor allem die Expression von α-SMA anstiegen ließ. Die Kombination aus beiden Ansätzen potenzierte die Ergebnisse. Antifibrotische Eigenschaften von HGF konnten im beschriebenen Modell bestätigt werden.

SCHLUSSFOLGERUNG: Das beschriebene Modell zeichnet sich durch eine, vollständig in vitro produzierte, repräsentative Extrazellulärmatrix aus. Dies bietet erstmals die Möglichkeit zur detaillierten Exploration von Narbengewebe in vitro, sowie zur standardisierten Testung neuer therapeutischer Ansätze. Die Entwicklung neuer Therapien für betroffenen Patienten könnte damit deutlich beschleunigt werden.

1 INTRODUCTION

During the last century the working environment and job requirements have undergone dramatic changes for the majority of people. In former days manual labour was the basis of everyday work for most employees, whereas nowadays an increasing number of jobs depend on communication skills, including voice, speech, foreign languages and not to forget hearing. This leads to an increasing number of patients dealing with various disorders in the aforementioned abilities, who seek for medical help, especially in the field of otorhinolaryngology.

Statistics from the US show that at the beginning of the last century around 80% used mainly their manual skills in everyday work. During the course of time this evolved to only 37% remaining on manual labour at the end of the last century, indicating more than a doubling of the amount of workers depending on communication (63%) [1]. In big cities like New York this fraction is even greater with around 88% [2]. As a consequence individuals with communication disorders are facing substantial difficulties in finding a job, resulting in an unemployment rate of 41.9% for affected persons [2]. Moreover these disabilities are associated with a lower social class and lower income of around 85% of healthy competitors [2].

When looking at the whole population, around 10% are affected by communication disorders, whereas voice and speech disorders account for around 3% [3]. Finally, estimates show that costs of communication disorders are around $186 billion dollars per year in the United States, which is around 3% of the Gross National Product, representing a significant economic loss [2]. Corresponding data for Europe or Austria, to the best of my knowledge don´t exist. Nevertheless it might be assumed that statistical data is comparable in Europe to the aforementioned, emphasizing the need for appropriate therapies for relevant disorders in this area. For patients with hearing impairment various effective treatment options already exist, including cochlea implantation, for which a decrease in habituation costs [4] and an increase in productivity [5] of affected individuals have already been shown. Similar results are

still missing for patients with voice disorders. Especially VF scarring remains one of the major burdens in modern laryngology with the lack of satisfying therapy regimes for dysphonic patients.

1.1 The human larynx

The larynx as primary organ for the production of voice is composed among others of various cartilaginous structures, numerous muscles and a right and left vocal fold (see figure 1). In a healthy larynx the interaction of these components enable two main positions of the VFs with an open glottis during inspiration and a closed glottis during phonation and swallowing (see figure 2). All muscles involved in the movement of the VFs from the lateral position during inspiration to the medial position during phonation are innervated by branches of the vagus nerve. The cricothyroid muscle is innervated by the external branch of the superior laryngeal nerve, whereas all the other involved muscles are innervated by the so called recurrent laryngeal nerve. As a consequence muscles that are responsible for opening and closing the glottis are innervated by the same nerve, what may cause potential difficulties in rehabilitation of patients with damage of this specific nerve, especially after thyroid surgery.

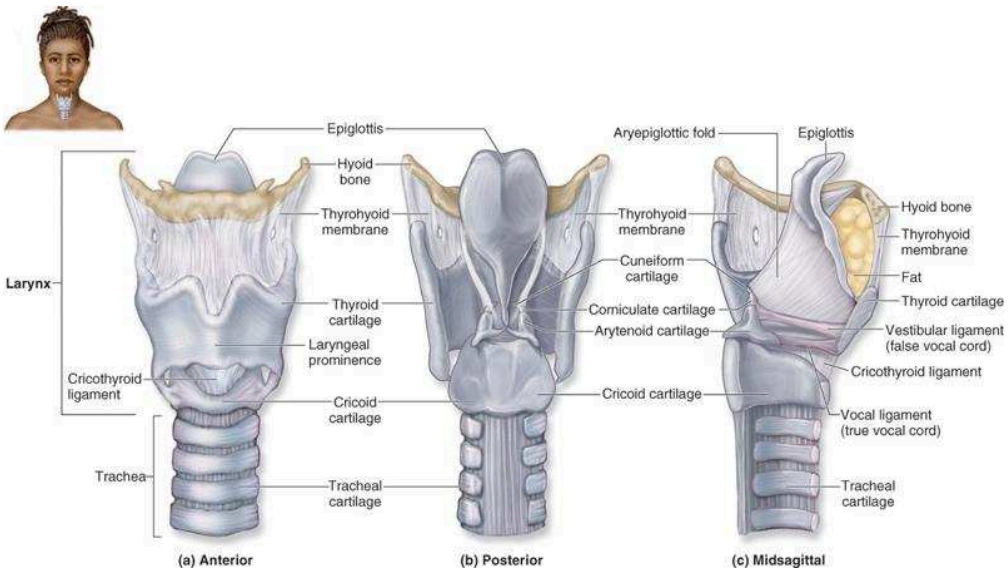

Figure 1: Human larynx in different views [6]

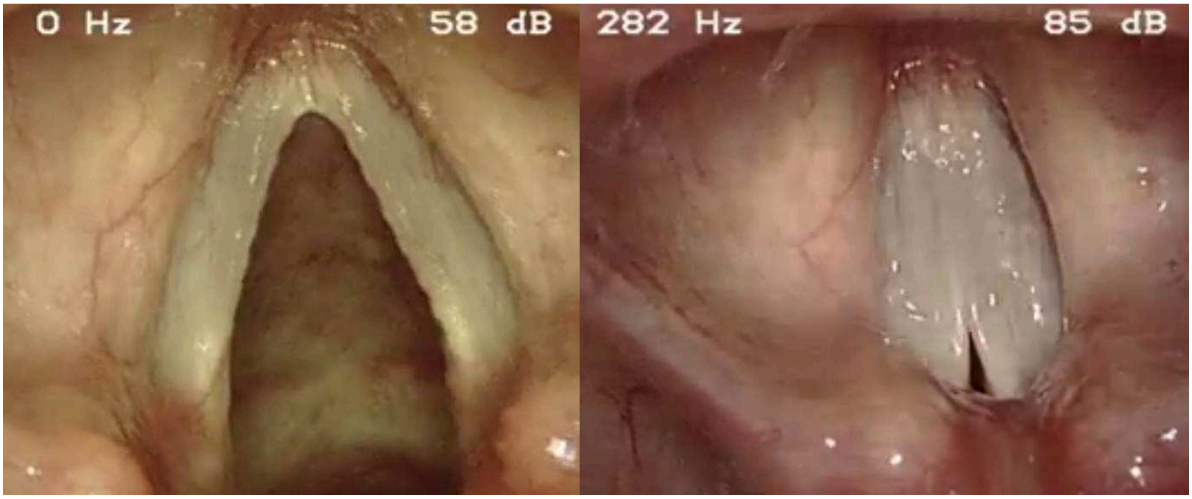

Figure 2: Endoscopic image of healthy vocal folds during inspiration (left) and phonation (right)

1.2 The human voice

The human voice is not only used to transport information through speech, but is also involved in the expression of many biological and psychological dimensions, including sex and age, body size and shape, hormonal condition, dominance, masculinity or femininity and attractiveness [7]. In this context, two key acoustic components could be defined, the fundamental frequency (F_0) and formants [8].

F_0 is determined by the oscillation of the vocal folds, elicited by the air, which is pressed through the closed glottis during phonation.

For listeners F_0 is recognized as voice pitch and influenced by the length and the tension of the vocal folds. Increasing length and decreasing tension lead to a drop of F_0 and lower perceived voice pitch. This is judged by listeners as strength, dominance and masculinity [7]. These stereotypes seem to be driven by anatomical conditions that influence F0, since men tend to have larger bodies, thus having also bigger larynges and vocal folds [9].

Formants are resonant frequencies of the supralaryngeal vocal tract, compiled of the pharynx and the oral and nasal cavities, whereas the dimension of these components

influences the development of formants. The resonant frequencies of an individual vocal tract are attenuated when sound is produced by the VFs, leading to perception of voice timbre [7].

Since long it is known that special vocal training can influence these two key components of voice production. Professional singers are able to increase their F0 and their formants to frequencies of over 1200 Hz, representing six times the average pitch of a woman´s voice [10]. On the other hand for example politicians, like Margaret Thatcher, lower their voice frequencies in extensive training to exude strength and power [7]. However a precondition therefore is a sufficient oscillation of the vocal folds, enabled by their delicate microstructure.

1.2.1 Microanatomy of the vocal folds

Figure 3 shows a frontal section of a human vocal fold, with the vocalis muscle as its main part. This muscle is a voluntary muscle and is innervated by a branch of the vagus nerve, named the recurrent laryngeal nerve and its final branch the inferior laryngeal nerve. By varying the strength of contraction of this muscle its mechanical characteristics can be altered with resulting effects on voice production. The elastic conus covers partly the vocalis muscle, consisting of mainly collagen fibers. Parts of these fibrous tissue fibers also grow into the muscle itself, resulting in a tight connection within these two structures. Often the elastic conus is also referred to as vocal ligament. Moreover it represents at the same time the deepest layer of the lamina propria [11].

The lamina propria consists mainly of extracellular tissue, a matrix of carbohydrates, lipids and particularly proteins. Studies have mostly concentrated on latter ones which are easy to investigate by histology. Thereby proteins could be divided in interstitial proteins like proteoglycans (esp. hyaluronic acid) and glycoproteins, as well as in fibrous proteins like collagens and elastins [12]. Furthermore the distribution of these fibrous proteins within the lamina propria establishes a tri-layered micro-architecture.

The superficial layer, often also referred to as Reinke´s space, is very loose with only few elastin and collagen bundles. Deeper in the intermediate layer mainly elastin fibers were identified and as mentioned above, the deep layer of the lamina propria is made up mostly by collagen bundles (see figure 4) [13].

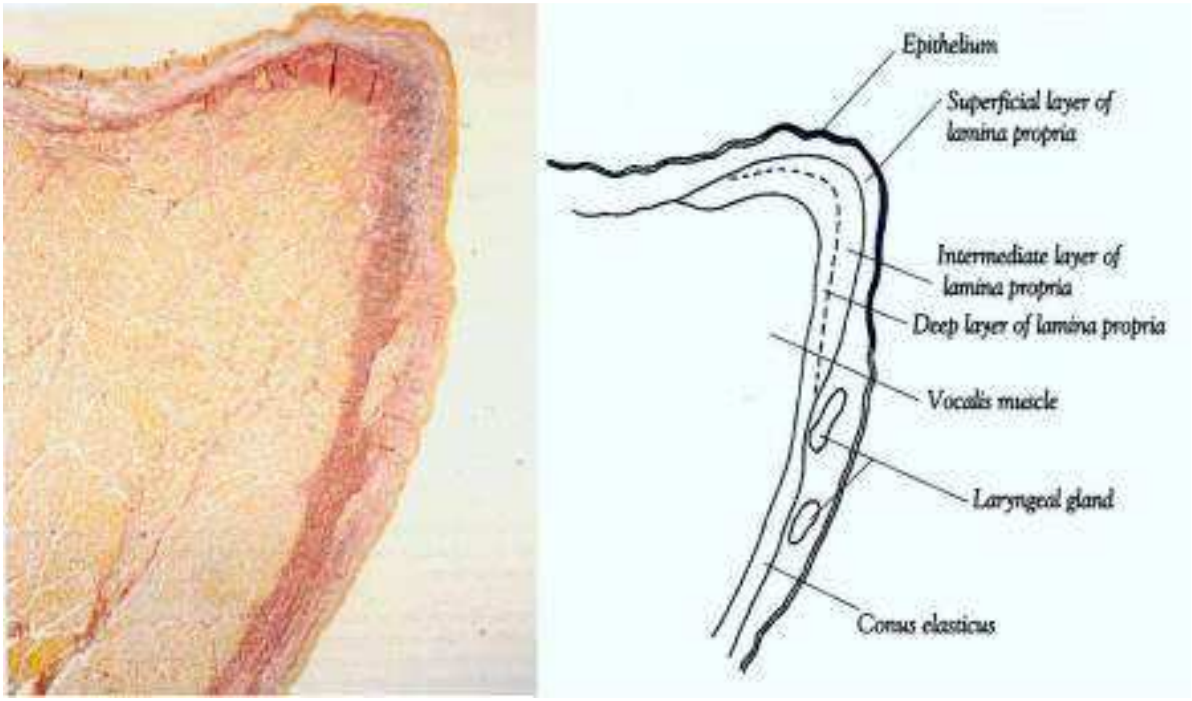

Figure 3: Frontal section of a human vocal fold; histological structure (left) schematic structure (right) [14]

These delicate histologic variations in the composition of the lamina propria have been studied extensively during the last decades and are crucial to physiology and pathology within the VFs. It has been shown for example that the arrangement of connective tissue fibers as outlined above is age-dependent in human vocal folds. In foetuses and neonates a vocal ligament could not be found and the layered structure of the lamina propria was seen to develop over time with a complete tri-layered appearance not earlier than 17 years of age [15]. Moreover during senescence cross-linking of elastin fibers seem to appear especially in the superficial layer of the lamina propria, combined with atrophy in this region [16]. In our group we could also show an

influence of age on glycoproteins like hyaluronic acid (HA). Levels of HA-production of fibroblasts obtained from old rats were decreased compared to samples from a young control group [17]. The importance of age and its influence on the extracellular matrix (ECM) has also been reported by Kohn et al. in different tissues [18].

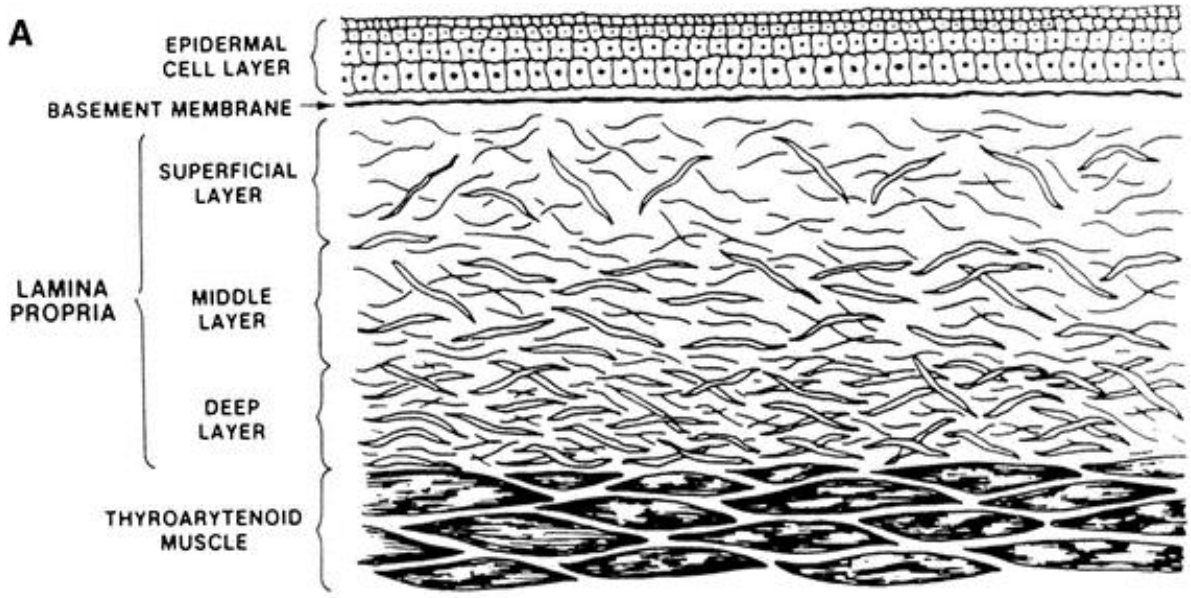

Figure 4: Layered structure of vocal folds [12]

The topmost layer is built by the epithelium, a multirow nonkeratinized squamous cell epithelium, providing essential mechanical support during phonation and coughing [19]. Although the lamina propria has been of major interest in most studies during the last decades, the importance of the epithelium has been discovered in the last few years. In 2015 a group from the Harvard Medical School revealed in an extensive investigation that within the epithelium three different layers can be distinguished with different expression of molecular markers (see figure 5). These results might be of upmost importance in carcinogenesis of the vocal folds [20].

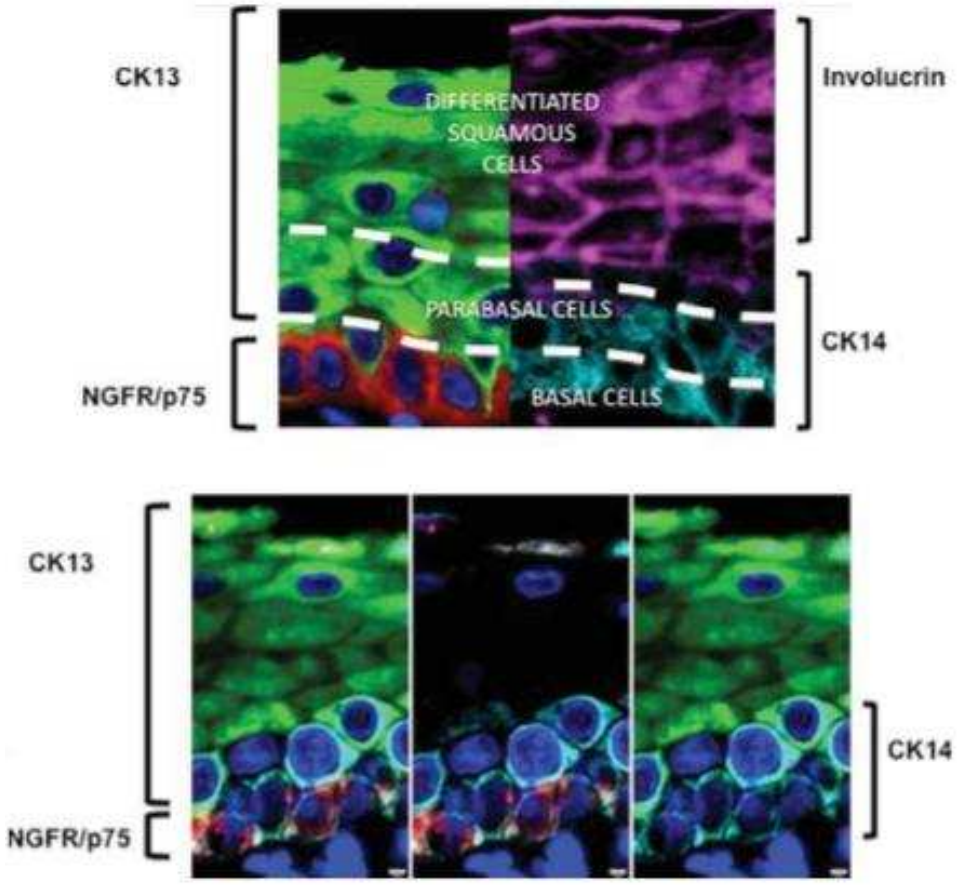

Figure 5: Distinct layers of vocal fold epithelium [20]

Moreover it has been shown in an excised human larynx model that an epithelium, acting as an intact surface layer is essential for normal symmetric vibration and glottal closure [21]. Also epithelial thickness seems to be important for physiology and pathology in this area [22]. A recent study by Goncalves et al. showed that in larynges of elderly people the epithelium was atrophic with only two to three overlapping cell layers compared to five to seven in the young control group [23]. This finding is likely to contribute to the development of poor voice in older persons, called presbyphonia.

The basement membrane zone can be found between the epithelium and the lamina propria, connecting these two regions with the help of anchoring fibres [16].

1.2.2 Cover-body theory

As early as in the 1970s Hirano and colleagues published their fundamental work on the histologic properties of the vocal folds and introduced the so called cover-body theory [11].

Due to this theory the cover consists of the epithelium and the superficial as well as the middle layer of the lamina propria, whereas the body is represented by the vocalis muscle and the deep layer of the lamina propria. During phonation the cover slides over the more rigid body, resulting in a typical mucosal wave [12].

Not to be seen by eye due to its high frequency, the mucosal wave could already be observed during early laryngologic research with high-speed cinematography [24]. An explanation for this complex mucosal movement with a vertical and a horizontal component was lacking till this cover-body theory was postulated. It was the first to provide sufficient explanation for this mucosal wave, especially in combination with the histological results of Hirano et al., showing evidence of free movement of the superficial tissue layers [11].

Furthermore not only physiological movements could be explained by this theory but also pathological conditions. Certain pathologies of the vocal folds are known to alter vibration in certain ways by changes in mechanical properties. Epithelial lesions like carcinomas, papillomas or hyperkeratosis for example lead to immobility in the affected regions by an increase of the elastic constant [25]. On the other hand oedema in the superficial layer of the lamina propria decreases the elastic constant, resulting in a pronounced movement of the mucosa. Another common disorder in laryngology is recurrent laryngeal nerve paralysis. Affected patients are unable to contract one or both VF; therefore the wave-like movement is missing. During the course of time muscle tissue is replaced by fibrous tissue if the paralysis persists, resulting in an atrophied vocal fold that moves like a flag in the wind during phonation [11].

1.3 Vocal fold scarring

In the majority of cases vocal fold scars arise from internal or external trauma to the larynx with a consecutive disruption of the layered structure of the VF. The underlying mechanisms are variable, ranging from external injury like blunt force trauma with fracture of the larynx to phonotrauma and iatrogenic lesions because of laryngeal intubation or phonosurgery. Especially professional singers or actors are in danger of phonotrauma by overusing their voice and applying high forces to their VF during demanding performances. Over time the career of such voice professionals can be threatened due to loss of function of their vocal folds by scarring conditions.

Iatrogenic lesions occur from traumatic laryngeal intubation when unusual high forces are applied or during long-time intubation with recurrent manipulations of the ventilation tube, resulting in mucosal damage. Moreover patients who undergo phonosurgery for several reasons like benign or malign tumors of the VFs are at risk for the development of scars postoperatively. Most of the tumors in this region arise from the epithelium and during phonosurgery tissue has to be removed to a various extent, depending on the depth of invasion by the underlying disease. One could assume, that the more tissue has to be resected the higher the degree of scar formation. In 2009 Hirano et al. published a paper on this topic on histologic results from biopsies of ten patients taken between three and twelve months after initial surgery. Depending on the type of cordectomy, indicating the depth of tissue removal, a trend to more pronounced scarring with deep resection was seen [26]. Especially when tissue had to be taken away down to the vocalis muscle excessive disorganized collagen occurred after wound healing resulting in a worse functional outcome. This underlines the demand for special expertise and the need to respect certain phonosurgical principles when performing this kind of procedures [27]. As the superior layer of the lamina propria is very trauma-sensitive and at the same time of enormous importance for vibration, tremendous care has to be taken to harm this area as little as possible to guarantee a satisfactory postoperative outcome [28]. This is even more important in patients with benign tumors, since a bad functional result after surgery in these cases cannot be accepted.

Furthermore chronic inflammation caused by smoking, other toxic inhalants, radiotherapy or laryngo-pahryngeal relux can also account for scar formation in vocal folds.

The aforementioned conditions are acquired reasons for vocal fold scarring. In a certain number of cases, although more seldom, this pathology arises congenitally. Affected patients suffer from dysphonia with onset during childhood and a combination with other malformations like webs or pathological vessels can be present. Moreover multiple lesions in both vocal folds and familial occurrence can be observed in these cases. Epidermoid cysts out of residuals of branchial arches are thought to be responsible for the development of different kind of lesions, depending on various rupture patterns (see figure 6) [29].

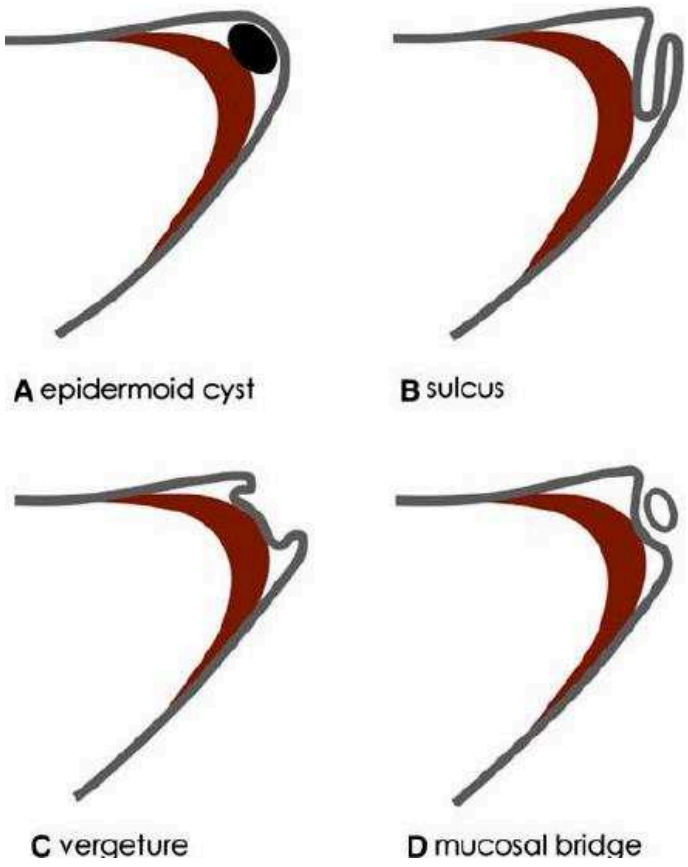

Figure 6: Congenital lesions of the vocal folds [30]

If the rupture of the cyst is above or directly at the free margin of the vocal fold a sulcus or a vergeture develops. If the cyst ruptures on both sides of the free margin a mucosal bridge will be the result (see figure 6 A-D).

Other authors hypothesized that sulcus vocalis could also arise from chronic inflammatory processes and/or local trauma, suggesting an acquired origin [31, 32]. This might explain especially the clinical experience that patients with sulcus often present during adulthood and not only childhood. Another theory suggests a summation effect of acquired and congenital mechanisms in the development of a sulcus [33].

1.3.1 Diagnosis

From a clinical point of view patients present in most cases with a long history of dysphonia. They complain of loss of vocal control and vocal fatigue, whereas a harsh, weak, breathy and little sustainable voice is evident. Like in every patient a complete medical history has to be gathered especially asking for any laryngeal trauma or surgery in this field.

Further on, auditory perceptual analysis, patient-perceived outcome measures like voice handicap index (VHI) and voice recording reveal valuable information.

Endoscopic investigation of the larynx has to be performed either with flexible or rigid endoscopes including especially stroboscopy to evaluate the mucosal wave properly. With a varying degree of disease the diagnosis can sometimes be hard to establish and a certain amount of experience is mandatory, especially in cases of sulcus, whereas in other cases the diagnosis is obvious like in patients with extensive scarring after cordectomy.

Important to notice during endoscopic and stroboscopic evaluation are two key features of vocal fold scarring, namely impaired vibration and insufficient glottic closure (see figure 7 for comparison of healthy and scarred vocal folds). Due to scar

formation especially in the lamina propria, the cover is not able to oscillate independently from the body any more, leading to a reduced or absent mucosal wave. In addition irregular and asymmetric vibrations can be observed. The insufficient glottic closure due to the scarring process is in most cases evident through a spindle-shaped glottis during phonation with a varying amount of air leakage. The amount of air leakage determines in most cases the degree of breathiness of the patient's voice.

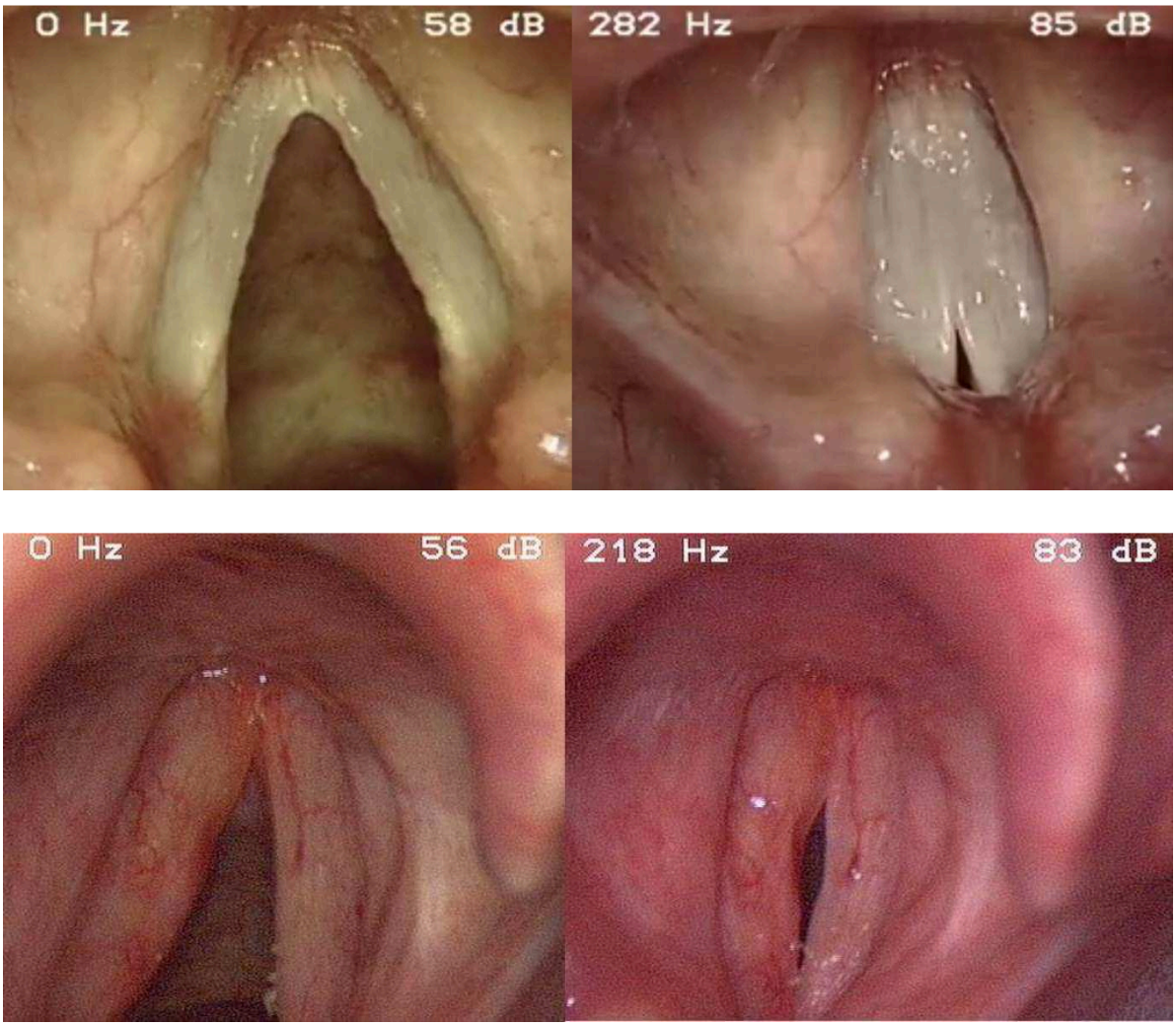

Figure 7: Healthy (upper row) and scarred (lower row) vocal folds during inspiration (left) and phonation (right)

Further important endoscopic findings of patients with vocal fold scars are compensatory hyperfunctional vocal behaviour like ventricular phonation or supraglottal squeezing, chronic inflammatory signs, thickening of the epithelium or dilated vessels on the surface of the vocal folds [34].

With the attempt to standardize the diagnosis of vocal fold scars Arens and Remacle introduced a classification of four different types [30]:

- Type I: Mild to moderate glottis insufficiency and reduced vibrations of the vocal folds. The scar involves the mucosal and submucosal levels of the vocal fold.
- Type II: Anterior moderate glottis insufficiency, seen around the anterior commissure region. The scar involves the vocalis muscle. No vibrations of the vocal folds.
- Type III: Considerable glottis insufficiency. The scar formation is adherent to the inner perichondrium and the cartilage. The defect extends up to the supraglottic region, twisted arytenoids may be noticed.
- Type IV: Considerable glottis insufficiency. Web formation at the anterior commissure region. Bilaterally reduced vibrations of the vocal folds.

Misdiagnosis might happen easily when patients are affected from only small lesions which are hard to see on endoscopic and stroboscopic examinations. Such patients are often misdiagnosed as functional dysphonia and referred to speech therapy with the lack of improvement after treatment. In these cases kymography or high-speed cinematography can enable proper diagnosis. Finally direct palpation of the vocal folds with microinstruments during microlaryngoscopy in general anaesthesia can assure difficult diagnosis like in vergeture or mucosal bridge (see figure 8) [33].

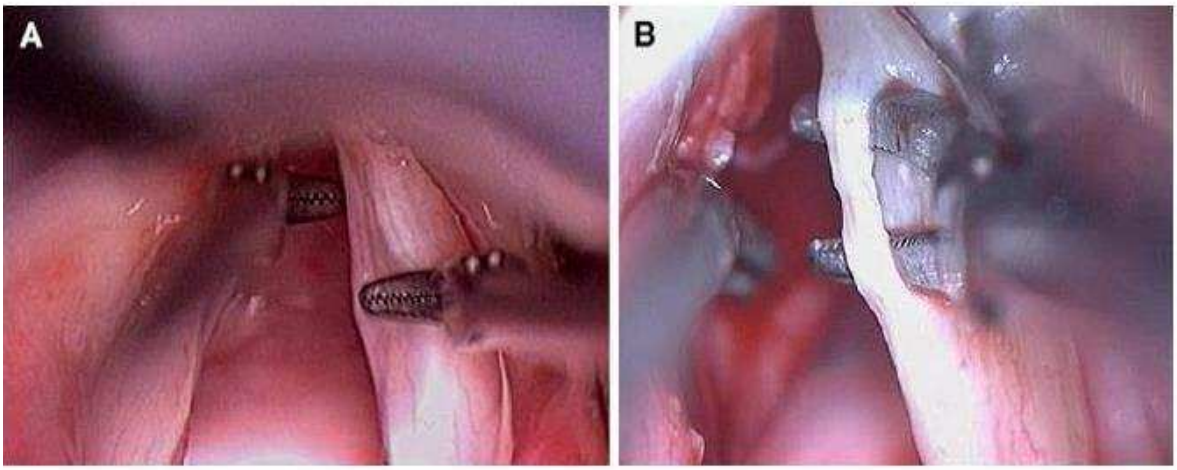

Figure 8: Vergeture (A) and mucosal bridge (B) [30]

1.3.2 Histology

When evaluating the histologic properties of fibrotic tissue like scars, it is the extracellular matrix (ECM) which is of upmost importance. This extracellular tissue is mostly built by fibroblasts and represents a matrix of proteins, carbohydrates and lipids. For the ease of histologic investigation, most studies in the last years have focused on the proteins in this compartment, which can be divided in fibrous and interstitial proteins. Either is important for physiology of the vocal folds [12].

Collagens and elastin are fibrous proteins, which are important to handle stress from a biomechanical point of view, by maintaining form and shape (see figure 9). On the other hand glycoproteins like fibronectin and proteoglycans like decorin as well as hyaluronic acid, a glycosaminoglycan, represent interstitial proteins. These molecular structures are located in the areas between fibrous proteins and are responsible for water content, tissue size, density of collagen fibers and tissue viscosity [35].

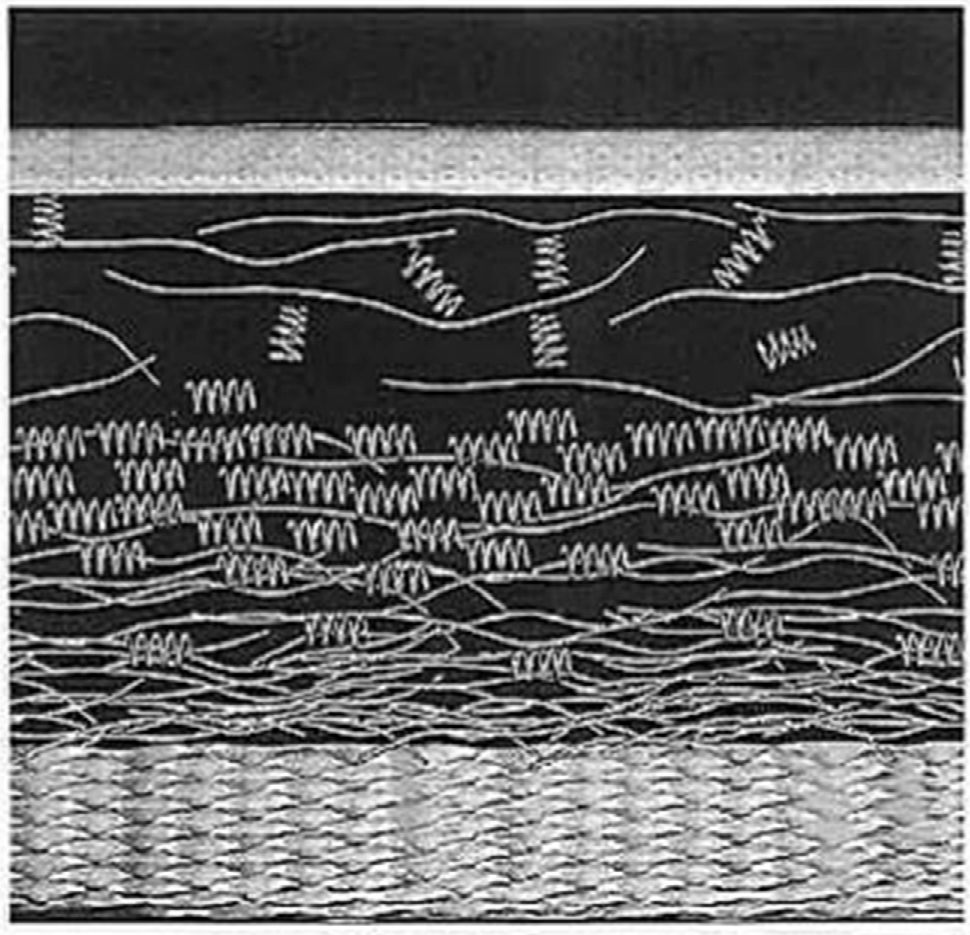

Figure 9: Arrangement of fibrous proteins in the lamina propria of vocal folds; ropelike struktures represent collagen, springlike structures represent elastin [12]

1.3.2.1 Collagen

In general around 20% of the human body is arranged by proteins. Collagen comprises more than 30% of that [36], whereas 19 different types of collagen have been described and further ten have collagen-like domains [37]. Nevertheless 90% of all the collagen in the human body is collagen type I, making it a ubiquitous molecule which is important for structural and functional integrity of most tissue [37]. Furthermore it is one of the most complex and largest macromolecules in the human body.

Collagen is a triple helix, formed by three polypeptide chains, each one coiled into a left-handed helix, whereas the three helixes are then wrapped around each other into a right-handed helix [38]. The final structure is often referred to as a rope-like rod (see figure 10) [37]. Hydrogen bonds and water bridges are responsible for further stabilisation of this structure, whereas side chains in different positions with hydrophobic or charged properties drive self-assembly, explaining the ability of polymerization in collagens [37]. Nevertheless the triple helix structure of collagen is very stiff which is necessary in some tissues like cartilage or bone, but might be disadvantageous when speaking of scarring processes.

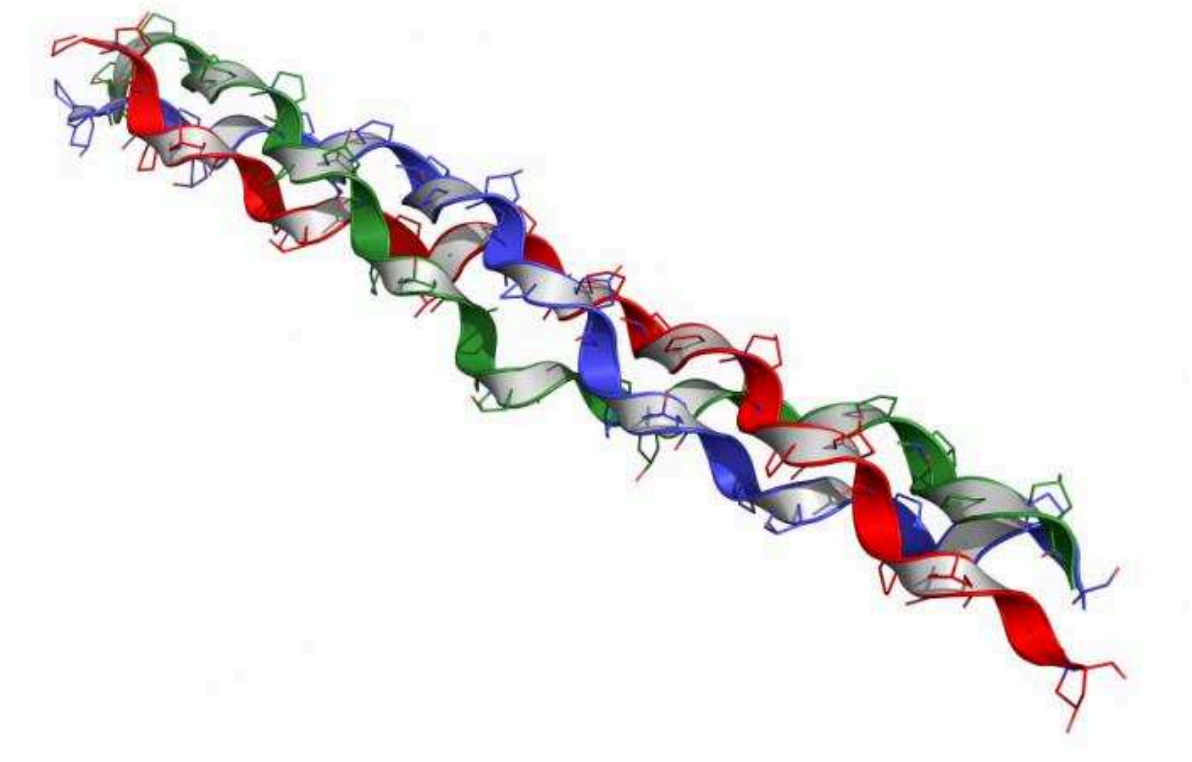

Figure 10: Structure of collagen [39]

Collagens can also be divided from a structural point of view, among others, into those which form network-like structures (type IV, VIII and X) and others which form fibrils (type I, II, III, V and XI) [37]; most important for scarring are the latter ones. Especially type I collagen seems to play a central role in the development of fibrosis, since it has been shown, that the strength of tissue increases with its collagen content and it was upregulated in various scar tissues [40].

The group of fibril forming collagens is synthesized as procollagens intracellularly and then secreted. Procollagen is a larger precursor of mature collagen. Generally type I procollagen is built up of two α1 and one α2 chains. In further procession the N- and C-propeptides of procollagen are cleaved by specific proteinases, namely the procollagen N- and C-proteinase. This step is already happening in the extracellular space. Further on the collagen is then self-assembling into fibrils, followed by cross-linking (see figure 11) [37].

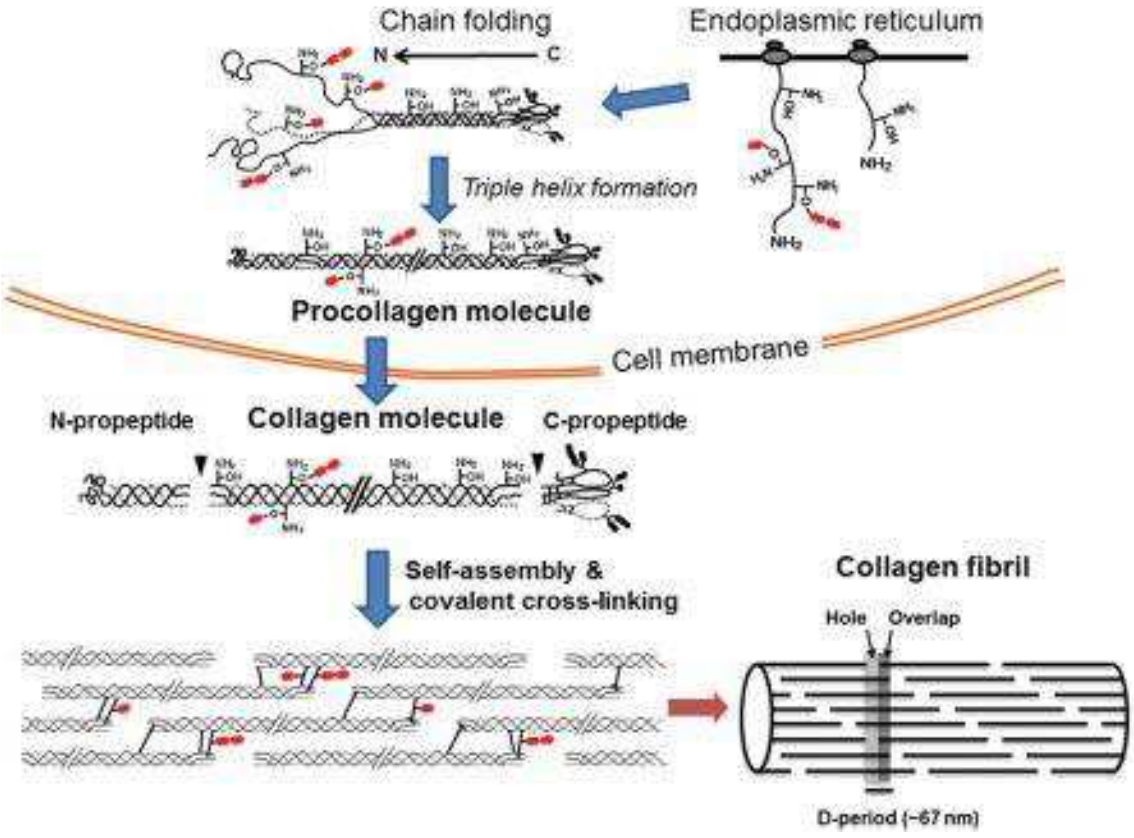

Figure 11: Intra- and extracellular procession of collagen [41]

Type 3 collagen is the predominant collagen in the lamina propria of healthy vocal folds. Moreover, collagen type 1 has also been detected in normal vocal folds, but to a lesser degree [42]. Type 1 seems to play a more important role in the scarring process. Nevertheless procollagen 1 has also been described in the superficial and the deep layer of the lamina propria [42]. In general collagen forms bundles in the physiologic lamina propria, running parallel to the free edge of the vocal fold [42].

1.3.2.2 Elastin

Elastin can be produced by different cell types like fibroblasts, chondroblasts or smooth muscle cells in a physiological state until puberty [43]. During senescence this protein becomes less functional and is normally not secreted any longer, with the exception of pathological circumstances like in hypertension [44]. Herein it has been shown that due to mechanical stress cells are starting to produce elastin again [45]. Nevertheless these tardy synthesized proteins were seen to be of less functional quality [44].

In general the function of elastin in tissue is to maintain the shape and manage mechanical load [46]. By forming elastic fibers it enables tissue to expand and recoil, whereas depending on the type of stress which the certain tissue has to sustain, different structures are built up by these fibers. In vessels for example elastic fibers are arranged in concentric rings. As mentioned above elastin is found in most tissues together with collagen to restrict mechanical stress and inhibit damage [44].

A cross-linked polymer of 72 kDa, which is insoluble and built up of tropoelastin monomers, represents the final elastin protein [44]. The elastin gene on the long arm of chromosome seven, which is one of the largest in the whole human genome, encodes for tropoelastin [44]. During further procession the enzyme lysyl oxidase is responsible for cross-linking of tropoelastin molecules, resulting in insolubility of the final elastin fiber [44]. When mechanical stress is applied each of the linked

molecules can expand and recoil, resulting in the ability of the whole network to stretch and retract as one unit (see figure 12).

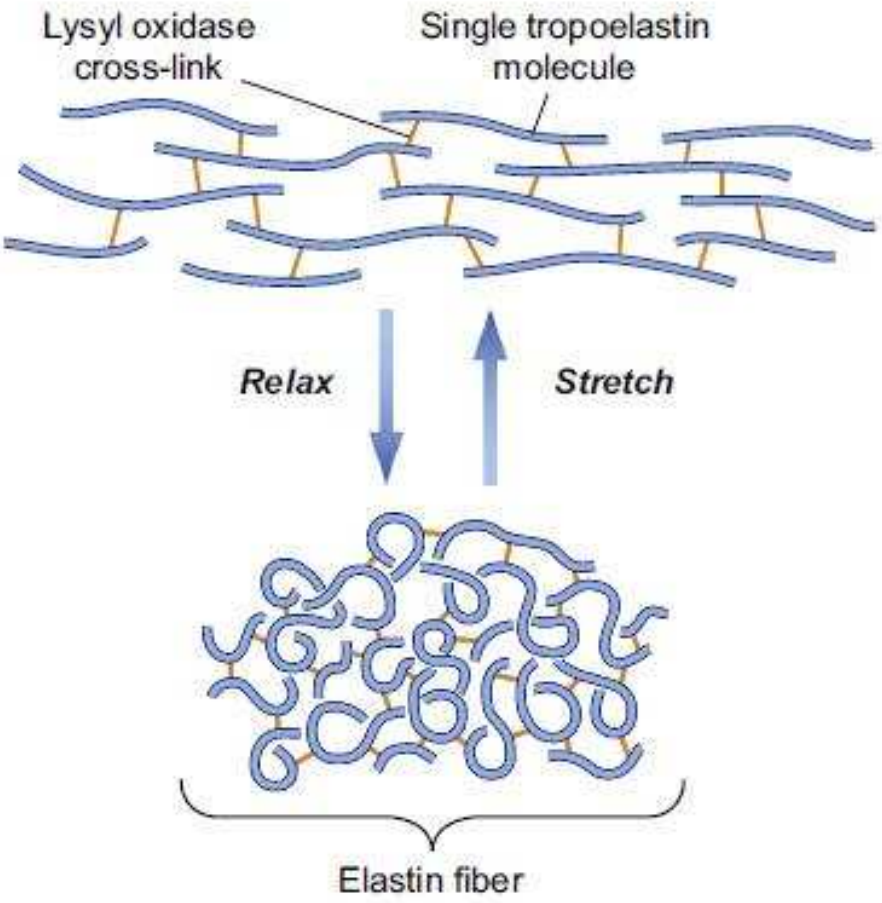

Figure 12: Schematic structure of elastin fiber [44]

In the composition of elastic fibers, elastin is essential in combination with a network of heterogenous microfibrillar proteins. During the development of tissue these microfibrillar proteins are secreted first into the extracellular area to build a certain scaffold. Tropoelastin molecules are then synthesized and also secreted into the extracellular space, where cross-linking with the help of lysyl oxidase takes place as described before (see figure 13 A). Further on the elastin molecules are included into the network of proteins, like cells into a scaffold (see figure 13 B). Finally the microfibrils combined with the mature elastin form the elastic fiber (see figure 13 C).

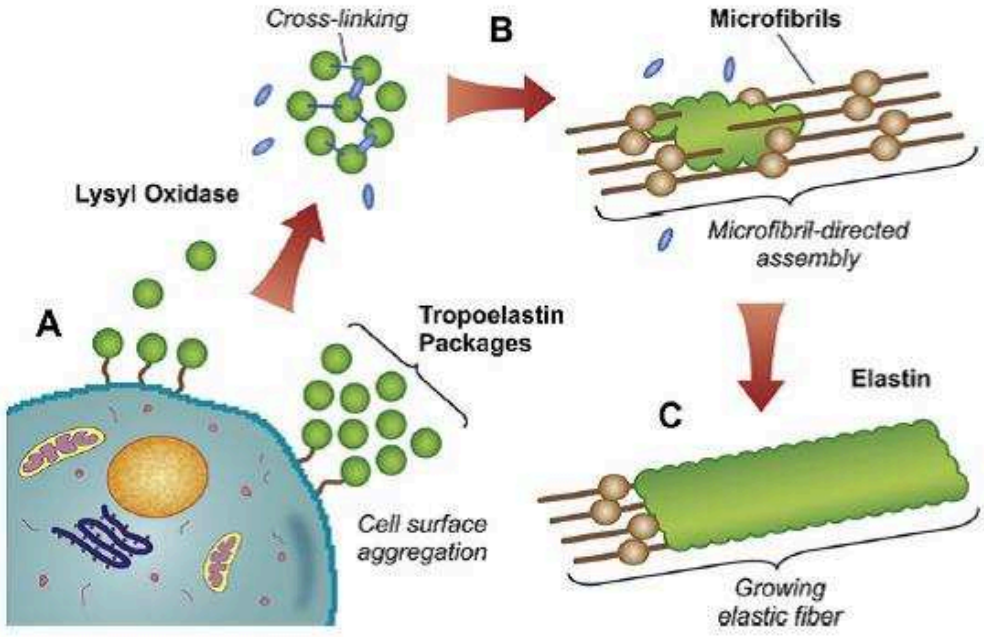

Figure 13: Concept of elastogenesis [46]

In the lamina propria of human vocal folds elastin was found in three different types, namely elastic fibers, elaunin and oxytalan, whereas the two last mentioned forms were often referred to as immature. This is no longer true, since they have been described as present in adult tissues too [13]. Looking at the distribution within the lamina propria, oxytalan and elaunin are found mostly in the superficial layer, whereas elastic fibers are increasing with depth in the intermediate and in the deep layer [47]. Evaluations of elastin content in the lamina propria depending on sex have been inconclusive over the last years with some authors describing more compact fibres in females [44], whereas others described no difference between male and female subjects at any time [48]. Moreover the results for elastin as a function of age are also incongruent. Thibeault and colleagues found increased mRNA levels for elastin in human vocal fold fibroblasts from aged donors compared to young subjects [49], but on the other hand a study with fibroblasts from rat vocal folds found a decrease of tropoelastin mRNA in aged specimens [50]. A possible explanation could be an age dependent increased cross-linking through oxidation, resulting in increased

elastin mRNA along with decreased tropoelastin mRNA, but this has to be evaluated further [44].

Finally from a clinical point of view it has been shown that individuals with Williams syndrome, a congenital disorder of elastin production, present with hoarseness and a instability in pitch compared to control [51].

1.3.2.3 Fibronectin

Fibronectin is a glycoprotein, which is involved in various processes in the human body. It acts in tissue as an important adhesion molecule. Especially in wound healing fibronectin plays a decisive role, because of its chemotactic effects for fibroblasts and inflammatory cells [52]. Moreover it is involved in the organization of extracellular matrix [52]. Fibronectin can be synthesized in two different ways. First it can be produced by the liver, from where it is secreted into the blood plasma in its soluble form. It contributes in blood coagulation and wound healing by binding fibroblasts and keratinocytes. Second, fibronectin can also be secreted by chondrocytes, macrophages or fibroblasts themselves in its insoluble form. This insoluble form of fibronectin gets incorporated into the extracellular matrix and is capable of building bridges between collagen fibrils and various other molecules of the ECM. Furthermore fibronectin acts in its insoluble form as adhesion molecule for different cell types during migration through tissue [53].

Different studies revealed that fibronectin represents a key factor in impaired wound healing. Kischer et al. demonstrated as early as in the 1980s that fibronectin is dramatically enhanced in hypertrophic scars and keloids in immunofluorescence staining compared to normal human dermis [54]. Moreover it has been shown, that fibronectin secretion is increased in fibroblasts from chronic wounds compared to fibroblasts from normal skin or acute wounds [55].

In normal, healthy vocal folds fibronectin has been detected especially in the superficial layer of the lamina propria as well as in the area of the basement membrane [56, 57].

1.3.2.4 Hyaluronic acid

Hyaluronic acid is another ubiquitous molecule in the human body, playing an essential role in space filling, wound healing, shock absorption and various other processes [58, 59]. From a biochemical point of view hyaluronic acid can be classified as a nonantigenic glycosaminoglycan polysaccharide, consisting of repeating chains of D-glucuronic acid and N-acetylglucosamine (see figure 14) [60].

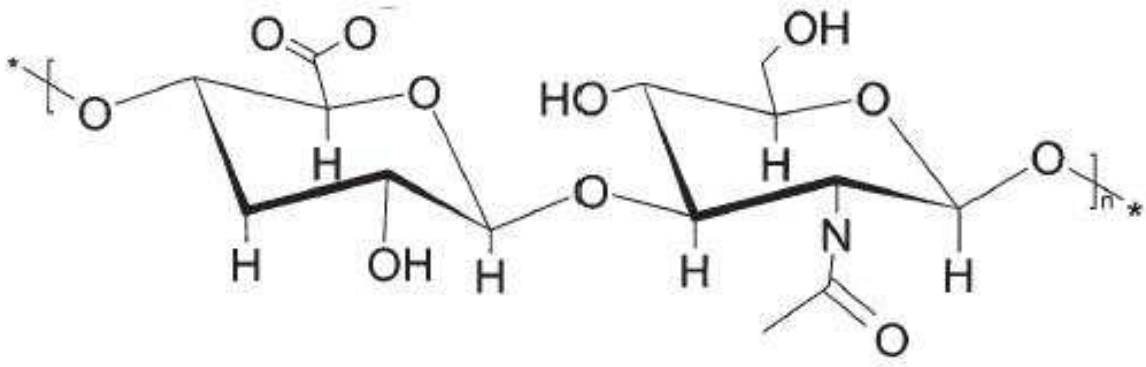

Figure 14: Structure of hyaluronic acid; D-glucuronic acid and N-acetylglucosamine [60]

To fulfil the above mentioned functions like shock absorption, an intensely hydrated gel can be formed by hyaluronic acid. This is enabled by many negative charges on the molecule of hyaluronic acid, attracting water and cations. Thereby hyaluronic acid is capable of filling a space a thousand times bigger than anticipated by its composition alone [60]. Especially in vocal folds this effect is crucial since it influences elasticity as well as viscosity, and as a consequence phonation itself [61]. In a study by Chan et al. the elasticity and viscosity of vocal folds have been

investigated in the absence and presence of hyaluronic acid [61]. They described that the viscosity of the vocal fold cover was increased and the elasticity was decreased in the absence of hyaluronic acid, resulting in far suboptimal preconditions for phonation.

In general hyaluronic acid can be found in the ECM of the entire lamina propria, with a little accentuation in the intermediate layer [59]. It has also been shown that the amount of hyaluronic acid is lower in vocal folds from women than from men [62]

As mentioned above hyaluronic acid is also essential in wound healing. Fetal scarless wound healing seems to be dependent on increased levels of hyaluronic acid in the wound fluid compared to adult wounds [63]. In dermal wounds it has also been shown that a hydrogel film of hyaluronic acid can enable vascularization, collagen organization and reepithelialisation [60].

1.3.2.5 Cells in the lamina propria

All the above mentioned components of the extracellular matrix are produced by VF fibroblasts (VFF), giving these cells a central role in the process of wound healing and fibrogenesis. In the human vocal folds cells are sparse in the middle portion of the vocal fold, whereas in the anterior and posterior portion the so called maculae flavae have been described (see figure 15) [64]. These elliptical shaped areas of 1.5x1.5x1mm in size are built by extracellular matrix and very dense masses of cells. Close investigations of the maculae flavae revealed that cells present in this area are mostly stellate shaped fibroblasts [65]. These newly described fibroblasts produce extracellular matrix constantly. In contrast conventional spindle-shaped fibroblasts, which are found in the rest of the vocal fold mucosa, are inactive under physiologic circumstances [66]. Recent studies on the maculae flavae showed moreover evidence for stem cells in this area [67]. These stem cells seem to be capable of migrating from the maculae flavae to the site of injury [68]

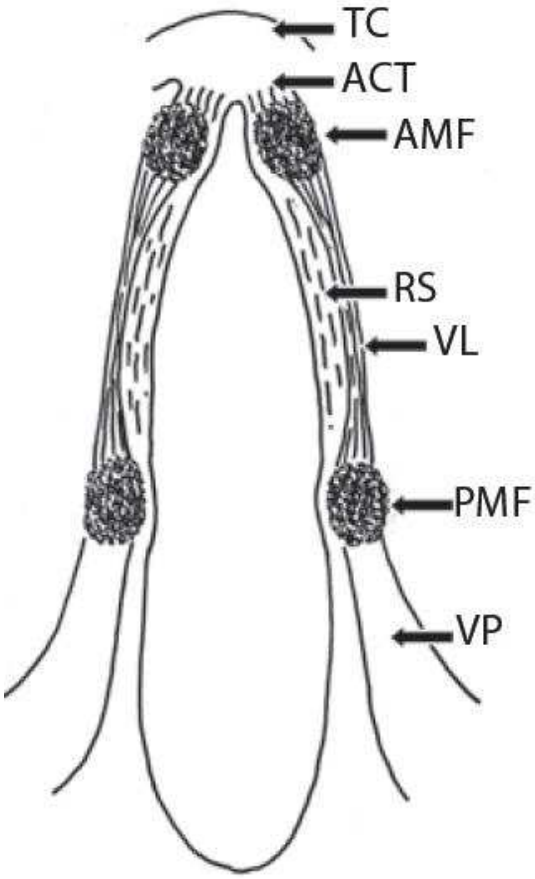

Figure 15: Schematic representation of the human vocal folds; AMF= anterior macula flava; PMF= posterior macula flava; RS= Reinke´s space; VL= vocal ligament; VP= vocal process of arytenoid cartilage; ACT= anterior commissure tendon; TC= thyroid cartilage; [69]

1.3.2.6 Current understanding of vocal fold scarring

A lot of work has been done during the last years in basic and clinical science to improve the understanding of scarring processes in vocal folds. Most studies were performed in certain animal models since trials in humans are difficult to perform. Studies focused especially on alterations in the above mentioned components of the extracellular matrix, occurring in wound healing and fibrogenesis during the course of time. Hansen et al. gave a comprehensive overview of histologic changes in different

components of the extracellular matrix after injury in different animal models (see figure 16) [70].

In a rabbit model it was shown that the collagen content was decreased two months after injury. Moreover the characteristic organization into bundles, as described before in normal vocal folds, was missing [42]. Six months after injury collagen levels were found to be increased compared to normal vocal folds, with the appearance of thick unorganized bundles [71]. The precursor of collagen, procollagen 1, showed an inverse behaviour with increased levels after two months and normal levels after six months [71, 72]. Elastin fibres were decreased, short and compact two months after injury, but increased to normal levels after six months, whereas fibres remained disorganized and fragmented [42, 71, 72].

	Injured Rabbit Vocal Fold						Injured Canine Vocal Fold		Injured Pig Vocal Fold			Injured Rat Vocal Fold			
ECM Component	**3 day**	**5 day**	**10 day**	**15 day**	**2 mo**	**6 mo**	**2 mo**	**6 mo**	**3 day**	**10 day**	**15 day**	**2 week**	**1 mo**	**2 mo**	**3 mo**
Procollagen					↑	↔	↑	↔							
Collagen					↓	↑	↔	↑	↔	↔	↑	↑	↑	↑	↑
Elastin					↓	↔	↓	↓							
Fibronectin					↑		↑	↑				↑	↑	↔	↔
Decorin					↓										
Fibromodulin					↓										
Syndecan-1							↔	↔							
Syndecan-4							↑	↑							
Cadherin							↔	↔							
Hyaluronan	↓	↔	↓	↓	↔	↔	↔	↔	↓	↓	↓	↓	↓	↓	↓

↑ = increased level; ↓ = decreased level; ↔ = no change; similar to control; blank = not measured.

Figure 16: Summary of histologic changes in vocal folds after injury [70]

In a canine model similar results could be observed for procollagen, collagen and elastin at two and six months after injury [72].

Looking at interstitial proteins, fibronectin was shown to be increased in rabbit and in canine vocal folds at all investigated time points [73, 74]. In rats fibronectin was also elevated in the early stage of wound healing but became normal after eight weeks post injury [75]. Moreover in a study by Hirano et al. a dynamic association between

collagen and fibronectin was described [76]. It was suggested, that substitution of fibronectin by collagen is a tardy process, since two months after injury fibronectin was increased but not collagen, whereas six months after injury both parameters were found to be elevated [76]. As a consequence it was postulated that targeting fibronectin especially in the later stages of wound healing could alter the production of collagen and thereby be a potential therapeutic target against the development of fibrosis [77]. Further studies are needed to prove this theory.

Hyaluronic acid was found to be decreased in the early stages of wound healing in different studies using rats, pigs and also rabbits [78, 79]. Other investigations, performed in the late stage of healing, revealed, that two and six months after injury the level of hyaluronic acid was similar to normal vocal folds [42, 71, 72]. The primary decrease in combination with the late normalization suggests that hyaluronic acid is important in the early phase of healing [70]. This has to be kept in mind especially when trying to develop new therapeutically approaches that could influence hyaluronic acid.

In 2010 Yamashita et al. introduced a new model, using mice [80]. They investigated histologic changes after unilateral vocal fold injury from day one until day 56 postinjury. Comparable to the models mentioned above they found an early upregulation of procollagen 1 followed by subsequent upregulation of collagen 1. Concerning elastin they did not report any difference compared to the uninjured side, which is inconsistent with other published data.

Only few reports exist about histologic properties of vocal fold scars in humans. In 2009 Zapater et al. conducted a study on tissue of 12 human vocal folds [81]. Biopsies were taken from patients at least eight weeks after cordectomy and showed increased collagen bundles throughout all layers of the vocal fold lamina propria. Between 12 and 20 weeks postinjury the scar was ‘stabilized’, as described by the authors, with no further alterations in the extracellular matrix [81]. Also in 2009 Hirano et al. investigated samples of ten vocal folds 3 – 13 months after cordectomy [82]. In this study they did also describe alterations in hyaluronic acid, elastin and fibronectin and not only collagen. A finding of upmost importance in this study was that changes

in the extracellular matrix depend on the degree of tissue damage. Deeper resections of tissue at cordectomy resulted in pronounced collagen abundance and increasingly disorganized elastin. The content of fibronectin and hyaluronic acid varied strongly between samples [82].

In summary, the scarring process seems to evolve from increased levels of procollagen 1 in the early stage of wound healing to thick unorganized bundles of collagen and fragmented and disorganized elastin fibers in the late stage. Moreover a long lasting increase in fibronectin could be responsible for excessive matrix reorganization in the late stage fibrosis, whereas hyaluronic acid seems to be important in the early days of wound healing.

The aforementioned histologic alterations in vocal fold scars are presumed to increase stiffness of the mucosa and to reduce mucosal pliability [40]. Thibeault and colleagues investigated the rheologic characteristics of vocal fold scars in a rabbit model [42]. They found, that the elastic shear modulus and the dynamic viscosity were significantly increased in the scarred vocal folds compared to the uninjured ones. The elastic shear modulus represents the requirement of a certain amount of shear stress to produce a given deformation. Thus a higher elastic shear modulus implies the need for higher shear stress to reach a certain deformation. The increase in dynamic viscosity means that the shear flow of tissue was reduced. These findings confirmed for the first time in laryngologic research the clinical experience that scarred vocal folds seem to be stiffer than normal vocal folds [34, 42].

As outlined above the development of vocal fold scars is a complex process that evolves over time. Therefore an ideal treatment for affected patients would have to address this complexity to restore function.

1.3.3 Current treatment options

Conservative treatment should be considered as a first option. Comprising of voice therapy, antibiotics and steroids an improvement in quality of voice can be achieved in some patients. Especially in the early management of scarring these conservative options can be helpful [34].

Nevertheless in the majority of cases conservative attempts are unsatisfactory for patients in the long run so that surgical treatment options may provide help. Before that, patients have to be informed thoroughly about the uncertainty of postoperative results and the prospect of success. Patients have to know that the chances of natural voice sound postoperatively are low and that the major aim is to improve loudness as well as to reduce air loss and vocal fatigue [28]. In general, surgery should not be performed prior to six months after injury to the vocal folds to ensure completion of the healing process [83]. When planning the surgical approach it has to be evaluated whether the complaints of the patient arise mainly from insufficient glottic closure or impaired pliability of the vocal folds. In patients with insufficient glottic closure various medialization procedures can be offered, whereas in patients with impaired pliability as the main feature of disease the rigidity is in the focus of surgery [30].

Medialization procedures aim to reduce the insufficient glottic closure during phonation, which can be achieved by injection augmentation or medialization thyroplasty. For injection augmentation various fillers can be instilled into different areas to medialize the vocal folds under general anaesthesia during microlaryngoscopy, or in an office-based setting under local anaesthesia (see figure 17).

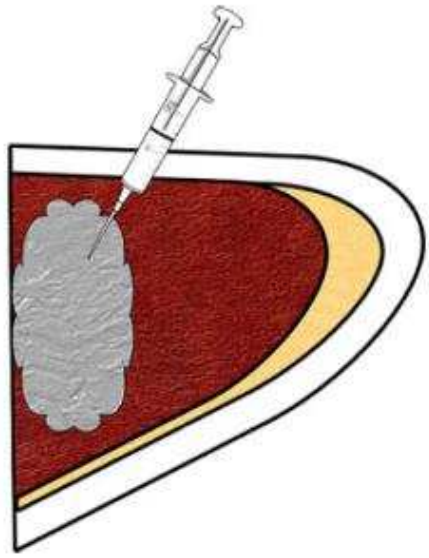

Figure 17: Concept of injection augmentation [30]

Fillers can be either autologous/xenogenic or alloplastic. The former have the advantage of biocompatibility since fat or fascia is commonly used [84]. On the other hand these materials undergo resorption leading to a decline of outcome in the long term, whereas the resorption rate differs from patient to patient. This makes it hard to predict how long the result will be satisfying for the patient, but another advantage of this attempt is that it can be repeated if necessary [85]. The latter, alloplastic fillers like silicone, do not present the disadvantage of resorption, which might be favourable in some patients showing stable long term effects. On the other hand this can also cause trouble, especially when the postoperative results are not as expected and the material has to be removed again. Moreover some patients developed foreign body reactions and granulations [86].

Medialization thyroplasty is a surgical procedure in which a window is excised from the thyroid cartilage, at the height of the vocal fold. Further on implants are inserted into the excised window to push the underlying tissue inward and to medialize thereby the vocal fold and reduce the glottic gap (see figure 18) [30].

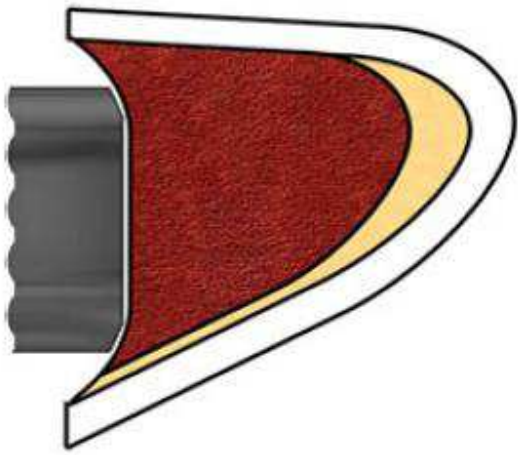

Figure 18: Concept of medialization thyroplasty [30]

A variety of implants for this purpose are available in different materials. Some of them are pre-fabricated in various shapes [87, 88, 89]. Most commonly used are silastic blocs, or titanium implants [87, 90]. One major advantage of this procedure is that the surface of the vocal fold itself does not have to be touched, hence avoiding injury to the mucosa and the risk of further scarring. Moreover medialization thyroplasty ensures permanent results since the implant can stay in place. In some patients this operation can even be performed under local anaesthesia, giving the surgeon the possibility to optimize the position of the implant while hearing changes in the patient's voice [91].

For cases in which rigidity is the main problem several techniques have been described in the literature attempting to restore the mucosal wave [29, 92]. All these procedures try to rebuild a gliding zone between the epithelium and the vocal ligament as described in the body-cover theory. However most described techniques demand a high degree of expertize whereas results are satisfactory in some cases but in the majority still not optimal.

One example for such a technique is the freeing of the epithelium described by Bouchayer and Cornut (see figure 19) [29, 93]. During this procedure the mucosa is

dissected from the underlying tissue (see figure 19 A) followed by the removal of scarred areas in the subepithelial layer (see figure 19 B). At the end the mucosa is flipped back and fixed again with fibrin glue or microsutures (figure 19 C) [29].

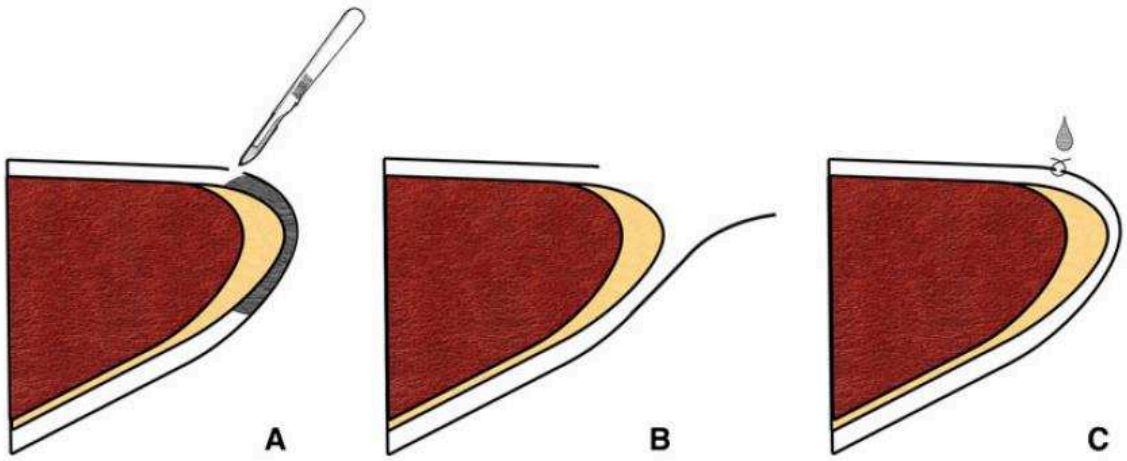

Figure 19: Freeing of the epithelium; A: Dissection fo epithelium B Resection of subepithelial scars C Turning back and fixation of the flap [30]

One can imagine that after this procedure the risk of scar reoccurrence is not negligible. To address this issue local injection of cortisone and the so called medial injection have been established. Medial injection means the instillation of fillers like autologous fat or hyaluronic acid into the subepithelial area to create a smooth gliding zone (see figure 20) [30].

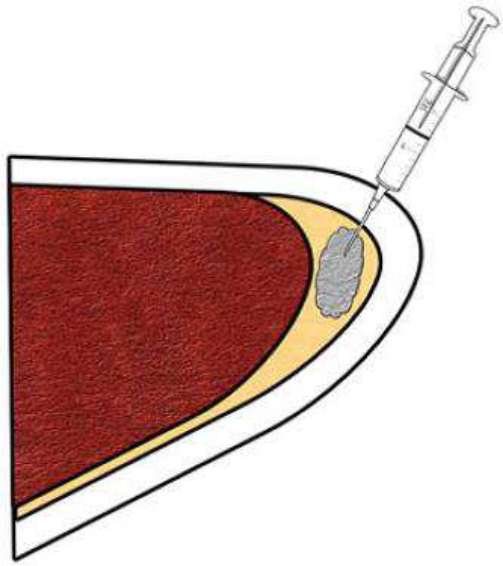

Figure 20: Medial injection of fillers to provide a new gliding zone [30]

Moreover different materials were proposed to be suitable for submucosal implantation, like gelatine sponge with fat, autologous veins or fascia [94, 95]. In some severe cases even free buccal mucosa grafting has been described [96]. During this procedure the scarred mucosa is removed from the larynx and a free mucosal flap from the buccal region is transplanted. When first published it was done in an open technique via a laryngofissure [96]. Over time endolaryngeal approaches for mucosal grafting have been developed but still it remains an effortful procedure, without the guarantee for reappearance of a mucosal wave postoperatively [30].

As outlined above, the structure of the lamina propria could not be restored yet despite enormous efforts to develop appropriate therapeutic concepts in the last years. However the reconstruction of the lamina propria remains the key to reconstitute a proper mucosal wave and to obtain optimal results for patients.

1.3.4 Tissue engineering as potential treatment option

Tissue engineering might be a promising solution in restoring the lamina propria of scarred VF. Especially cell therapy and treatment with growth factors have already been investigated in first experimental and pre-clinical trials, showing promising results [97, 98]. However, until these attempts will be available in clinical routine several obstacles have to be overcome. One major problem is that trials in humans are delicate. Every biopsy that is taken from a vocal fold represents another injury to the lamina propria and may thereby cause further scarring. Moreover safety of growth factors and stem cells will have to be proven first. As a consequence most trials have been performed in different animal models [98, 99]. It has to be mentioned that anatomy and physiology of the vocal folds is different in most animals than in humans. Moreover trials in an animal model are costly and time consuming since it takes at least six months to create a mature scar. In a first step an injury has to be established, whereas potential therapeutic agents should not be administered prior to half a year of healing period to get representative results.

Another option is cell culture, providing the possibility of closely investigating fibroblasts, the main player in fibrogenesis. Culturing fibroblasts does not take much effort and several studies which used vocal fold fibroblasts exist [100]. On the other hand fibroblasts do not produce a significant amount of extracellular matrix under conventional culture conditions and investigating fibrous proteins from supernatants is hardly representative. With the new technique of macromolecular crowding fibroblasts are stimulated to lay down a mature extracellular matrix to be investigated in vitro as will be explained.

1.4 Macromolecular crowding

Cell culture has been developed and refined with a lot of effort during the last decades. With these improvements a variety of whole new trials has been enabled, facilitating the close investigation of specific cells. Nevertheless, relevant differences exist when comparing physiologic conditions of cells incorporated in their natural tissue environment with conditions of cells in conventional cell culture. One of the major differences is that natural tissues represent a highly crowded surrounding with a lot of macromolecules like carbohydrates, lipids and proteins that occupy space in- and outside the cells. Depending on the investigated tissue a protein content of the extracellular space of up to 80g/L has been described [101]. In contrast, cells in conventional cell culture grow in monolayers surrounded by big amounts of salt water enriched with fetal bovine serum. The combination of fetal bovine serum, salt water and other additives results in a protein concentration of maximum 16g/L in the surrounding of cultured cells. From a clinical point of view this condition would be referred to as oedema or effusion [102].

In 1993 Zimmerman et al. already postulated that the differences in crowding features between the in vivo and the in vitro situation have to be considered in the evaluation of biophysical and biochemical processes [103]. Further on it has been shown that crowding influences among others protein conformation and stability, as well as the catalytic activity of enzymes [104, 105, 106].

Procollagen C-proteinase (PCP) is an enzyme which is responsible for cleavage of the C-terminal end of procollagen 1, the precursor of collagen 1. It is well known that PCP shows only minimal activity under non-crowded conditions [107]. As a consequence the majority of procollagen that is produced by fibroblasts in vitro accumulates in the supernatant rather than being further processed by PCP and incorporated into an extracellular matrix. Finally procollagen is discarded with the supernatant every time the culture medium is changed [108].

Introducing macromolecular crowding into cell culture could help to overcome the aforementioned issues in fibrosis research. Thereby inert synthetic or natural

macromolecules are added to the existing in vitro system to generate the so called excluded volume effect (EVE). The amount of excluded volume depends on the size of the macromolecules as well as on the unavailable space between the macromolecules (see figure 21) [109]. Proteins, carbohydrates or lipids can be used as macromolecules that occupy space but they should not interfere with the investigated reaction. From a thermodynamical point of view, the exclusion of volume decreases the conformational and configurational freedom (entropy), leading to increasing basal free energy of reactant molecules and upregulated enzyme activity [102]

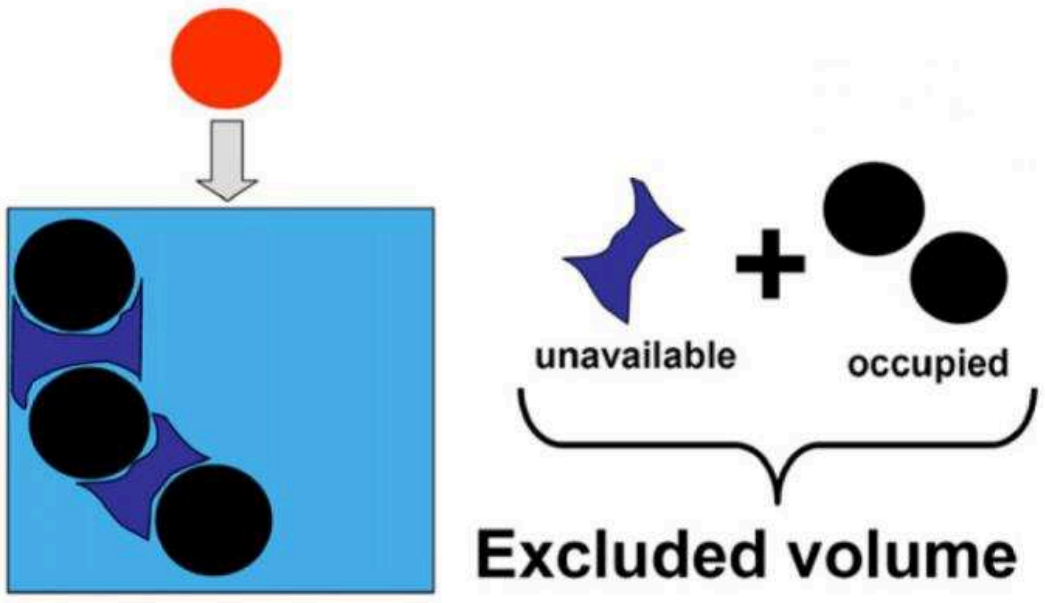

Figure 21: Excluded volume effect; red circle: test molecule, which is introduced into a crowded system with inert macromolecules (black circles) [102]

It is important to mention that simply reducing the amount of cell culture medium does not lead to the same results, as has been shown by Chen et al. [102]. The thermodynamic activity of a system is calculated by the total volume divided by the available volume in the system. A reduction of culture medium would accordingly lead to a reduction rather than to an increase of thermodynamic activity (see figure 22).

Moreover a reduced amount of medium is often not tolerated by the cultivated cells. Since the reduction of medium represents a decline in nutrients it results in most cases in early cell death.

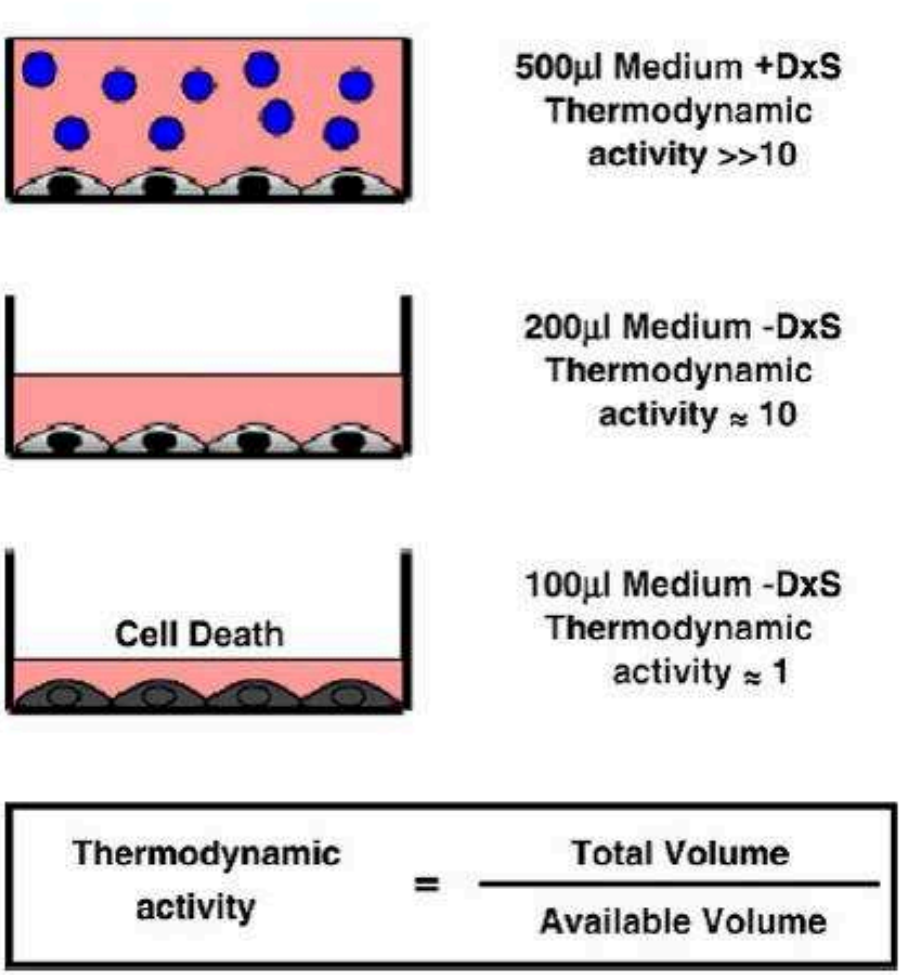

Figure 22: Thermodynamic activity of crowding vs. culture medium reduction; DxS represents inert macromolecules; the middle row is conventional cell culture, the upper row represents crowded cell culture and the lower row cell culture with reduced amount of medium [102]

The aim of this work was to develop a new model for vocal fold scarring, which would enable extensive investigation of extracellular matrix produced in vitro by vocal fold fibroblasts. Moreover it should provide the possibility for rapid testing of potential antifibrotic therapies.

2 MATERIAL AND METHODS

2.1 MMC in lung fibroblasts

Most work in the field of macromolecular crowding has been done by a group at the National University of Singapore [107, 110, 111]. After approaching M. Raghunath, the leader of this working group, I decided to serve an internship in his lab to get used to the implementation of this concept. During my work in his lab I was able to test different crowding approaches in different immortalized human lung fibroblast cell lines.

Employed cells were either WI-38 or IMR-90, both well-characterized normal human embryonic cell lines. Most work of this group has been done with either of these cell lines, as they grow reliably for a number of passages and deposit substantial amounts of collagen. Cells were obtained from American Tissue Culture Collection (CCL-75, Manassas, VA, USA).

Fibroblasts were seeded on 24-well plates (Nalgen Nunc International, NY) at 50 000 cells per well in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum (FBS, Sigma-Aldrich St. Louis, MO) and 1% Penicillin/Streptomycin (P/S) at 37°C with 5% CO_2. After 24 hours for attachment, medium was changed to serum free DMEM with 1% P/S for starvation. After another 24 hours cells were allocated to different groups by changing media and adding different agents (see table 1).

Group Ia **Control / standard medium**	**Group Ib** **Control / crowded medium**
Group IIa **TGFβ1 / standard medium**	**Group IIb** **TGFβ1 / crowded medium**
Group IIIa **HGF / standard medium**	**Group IIIb** **HGF / crowded medium**
Group IVa **TGFβ1 HGF / standard medium**	**Group IVb** **TGFβ1 HGF / crowded medium**

Table 1: MMC in lung fibroblasts; cell culture conditions

Standard medium consisted of DMEM with 0.5% FBS, 1% P/S and 100µM L-ascorbic acid 2-phosphate, since the formation of collagen is highly dependent on this vitamin [112]. Crowded medium consisted of standard medium and a mixture of neutral 70 and 400 kDa Ficoll™ (Fc) (37.5 mg/mL Fc 70 with 25 mg/mL Fc 400) (Sigma-Aldrich St. Louis, MO).

Moreover 5 ng/mL transforming growth factor beta 1 (TGFβ1) (Sigma-Aldrich St. Louis, MO) was added to certain groups, due to its ability to induce transformation from fibroblasts to myofibroblasts in vitro [113]. Finally hepatocyte growth factor (HGF) (Sigma-Aldrich St. Louis, MO), known for its antifibrotic properties [114], was added at 40ng/ml in certain wells.

2.1.1 Immunocytochemistry

After different culture periods (three, five and ten days) samples were harvested and Immunocytochemistry (ICC) was performed. Cell layers were fixed with methanol and blocked with 3% bovine serum albumin in phosphate-buffered saline (PBS) (Sigma-Aldrich St. Louis, MO). Primary antibodies were incubated for 1.5 h followed by

washing three times with PBS. Secondary antibodies and 4′,6-diamidino-2-phenylindoldilactate (DAPI) (Molecular Probes, Eugene, OR, USA) were incubated for 30 min and washed three times with PBS. Antibodies used were mouse anti-human collagen I (Sigma-Aldrich St. Louis, MO) and rabbit anti alpha smooth muscle actin (α-SMA) (Sigma-Aldrich St. Louis, MO), along with AlexaFluor 594 goat anti-mouse (Molecular Probes, Eugene, OR, USA) and AlexaFluor 488 chicken anti-rabbit secondary antibodies (Molecular Probes, Eugene, OR, USA). Cell nuclei were stained with DAPI. Pictures were taken with a Nikon TE600 fluorescence microscope.

2.2 MMC in primary rat vocal fold fibroblasts

2.2.1 Generating primary vocal fold fibroblasts

To transfer MMC to the vocal fold level, primary animal cells, vocal fold fibroblasts (VFF) of rats were used in a next step. Sprague-Dawley rats were chosen as appropriate source for primary vocal fold fibroblasts (pVFF) since the vocal folds (VFs) of rats present a tri-layered structure as do human vocal folds [115]. Moreover, VFF of Sprague-Dawley rats were successfully used in other in-vitro trials by our group [116]. Male Sprague-Dawley rats, aged 5 months were humanely euthanized by intracardiac injection of ketamine 100mg/ml after sedation with isoflurane (5%) in an induction chamber with an oxygen flow of 1.5l/min. After that, the larynges were excised. Under stereo-magnification, epithelium and underlying lamina propria of both VFs were carefully harvested. Specimens were minced with a sterile scalpel blade and placed into conventional six-well-plates (Nalgen Nunc International, NY). Growth medium consisted of DMEM (Life Technologies, Gaithersburg, MD) enriched with 10% FBS (Sigma-Aldrich St. Louis, MO) and 1% P/S. After confluence of 90%, pVFF were passaged using 0.5% trypsin. Cells at passage 3 were used for the experiment.

2.2.2 Macromolecular crowding

pVFF were seeded at a concentration of $5x10^4$ cells into 24 well plates (Nalgen Nunc International, NY) in DMEM enriched with 10% FBS and 1%P/S, 5% CO_2 at 37°C. After 24 hours for attachment, medium was changed to serum-free medium for starvation. Another 24 hours later, wells were allocated to different groups, analogous to lung fibroblasts as described above, by changing medium again (see table 2 for composition of different groups). Each group consisted of three replicates. For the control group DMEM containing 0.5% FBS, 1% P/S, and 100mM of L-ascorbic acid 2-phosphate was used. TGFβ1 (Sigma-Aldrich St. Louis, MO) was added to achieve myofibroblast differentiation, as described above. Crowded conditions were generated by addition of a mixture of 37.5mg/mL 70kDa Fc (Sigma-Aldrich St. Louis, MO) with 25mg/mL 400kDa Fc. Furthermore TGFβ1 and macromolecules were combined in one group.

Group Ia **Control / standard medium**	**Group Ib** **Control / crowded medium**
Group IIa **TGFβ1 / standard medium**	**Group IIb** **TGFβ1 / crowded medium**

Table 2: MMC in pVFF; cell culture conditions

To test an antifibrotic agent, HGF was chosen, as a well described growth factor with antifibrotic properties shown by restoration of fibrosis in several organs like liver, lung and kidney in animal trials [114]. Moreover, it was able to inhibit fibroblast to myofibroblast differentiation, induced by TGFβ1, in concentrations of 20 and 40ng/ml in an in vitro trial with VFF performed by other researchers [113]. So HGF (Sigma-Aldrich St. Louis, MO) was added at a concentration of 40ng/ml simultaneously with TGFβ1 and macromolecules in another group.

After 5 days, supernatants were sampled and immediately stored at -80°C. The cell layer was detached and lysed using cOmplete Lysis M (Roche Applied Sciences, Basel, SUI, Indianapolis, IN) and stored at -80°C.

2.2.3 Quantification

Assessments of collagen-I-α, hyaluronic acid and fibronectin levels from supernatants and cell layers were carried out separately using enzyme-linked immunosorbent assays (ELISA) kit for collagen-I-α (USCN Life Science Inc., Houston, TX, USA), rat-fibronectin ELISA (from ALPCO, Salem, NH, USA) and Quantikine ELISA for hyaluronan (R&D Systems, Minneapolis, MN, USA). α-SMA is a commonly used marker for myofibroblast formation and an essential part of the cytoskeleton. As such, ELISA was performed only from the cell layer (α-SMA ELISA by Cusabio, Wuhan, PRC). All assays were performed in duplicates (technical duplicates).

2.2.4 Immunocytochemistry

For immunofluorescence fibroblasts were grown on glass chamber slides (Lab-Tek II, Nalgene Nunc International, Naperville, IL, USA) at a concentration of $5x10^4$ cells per chamber and treated for allocating to different subgroups as described above. After five days slides were washed with PBS before being harvested. Slides were air-dried overnight and stored at -20°C. Cells were immuno-labeled using the UltraVision LP Detection System (Thermo Scientific, Fremont, CA) according to the manufacturer's instructions. The following antibodies were diluted in antibody diluent (Dako, Glostrup, Denmark) and applied for 30 minutes at room temperature: collagen-1-α (rabbit IgG, 8.5 mg/ml, Proteintech, Chicago, IL); α-SMA (goat IgG, 20 mg/ml, LifeSpan BioSciences, Seattle, WA). Cells were washed three times with PBS followed by incubation with the secondary antibody (Alexa Fluor 555 Donkey Anti-goat IgG, 10 mg/ml, Alexa Fluor 488 Donkey Anti-rabbit IgG, 10 mg/ml; both Life Technologies, Carlsbad, CA) for 30 minutes. Slides were again washed with PBS, mounted with ProLong Gold antifade reagent (Life Technologies, Gaithersburg, MD, USA) and

observed with a Leica DM600B fluorescent microscope (Leica, Wetzlar, Germany) connected to an Olympus DP72 digital camera (Olympus, Tokyo, Japan).

2.3 MMC in human vocal fold fibroblasts

Immortalized human vocal fold fibroblasts (hVFF) could be obtained from the lab of a collaborating group lead by S. Thibeault [117] and were used for experiments from passage 8-10. Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS (Sigma-Aldrich St. Louis, MO) and 1% P/S was used as standard growth medium again.

Cells foreseen for SDS-PAGE and Western blot were seeded at a concentration of 60.000 cells per well into 12-well-plates (Nalgen Nunc International, NY). For immunofluorescence cells were grown on glass chamber slides (Lab-Tek II, Nalgene Nunc International, Naperville, IL, USA) at 25.500 cells per chamber. During the first 24 hours after seeding attachment in standard growth medium, at 5% CO_2 and 37°C was enabled, followed by another 24 hours of starvation in FBS-free medium.
Cells were then allocated to different treatment groups by changing medium again. For the control group DMEM enriched with 0.5% FBS, 1% P/S, and 100 mM of L-ascorbic acid 2-phosphate was used, this is also referred to as standard medium. To induce myofibroblast differentiation 5 ng/mL transforming growth factor beta-1 (TGFβ-1) (Sigma-Aldrich St. Louis, MO) was added, as described earlier. "Crowded" conditions were generated in another setting by adding inert macromolecules, consisting of a mixture of 37.5 mg/mL 70 kDa Fc (Sigma-Aldrich St. Louis, MO) with 25 mg/mL 400 kDa Fc [118, 119]. Moreover, TGFβ-1 and macromolecules were added simultaneously in one group, representing the condition of maximal fibrosis.
To test antifibrotic compounds HGF (Sigma-Aldrich St. Louis, MO) and Botox Type A (IPSEN Pharma, Germany) were added in different concentrations to groups with TGFβ-1 and macromolecules. HGF was added at 40, 100 or 200ng/ml, Botox at 40 or 80IU/ml. After five days of incubation samples were further processed.

2.3.1 Immunocytochemistry

Fibroblasts were washed twice with phosphate buffered saline, fixed with methanol (-20°C), air dried for 30 minutes and stored at -20°C. 30 minutes prior to immunofluorescence staining cells were thawed and blocked with 3% BSA (Sigma Aldrich, Missouri, USA) for 1 h. Subsequently cells were immune-labelled using the UltraVision LP Detection System (Thermo Scientific, Fremont, CA, USA). The following antibodies were diluted in antibody diluent (Dako, Glostrup, Denmark) and applied for 90 minutes at room temperature: α-SMA (goat IgG, 20 mg/ml, LifeSpan BioSciences, Seattle, WA, USA); collagen-1 (mouse IgG, Dilution 1:1000, Sigma Aldrich, Missouri, USA, product no. C2456); fibronectin (rabbit IgG, 0.56µg/ml, Proteintech, Chicago, IL, USA). Cells were washed three times with PBS followed by incubation with the secondary antibody (Alexa Fluor 555 Donkey Anti-goat IgG, 10 mg/ml; Alexa Fluor 488 Donkey Anti-mouse IgG, 10 mg/ml, Alexa Fluor 488 Donkey Anti-rabbit IgG, 10 mg/ml; all from Life Technologies, Carlsbad, CA; USA) and DAPI (Life Technologies, Carlsbad, CA, USA) for 45 minutes. Slides were washed again with PBS, mounted with ProLong Gold antifade reagent (Life Technologies, Gaithersburg, MD, USA) and observed with a Leica DM600B fluorescent microscope (Leica, Wetzlar, Germany) connected to an Olympus DP72 digital camera (Olympus, Tokyo, Japan).

2.3.2 Cell count analysis after HGF treatment

For quantitative analysis of cells treated with HGF, chamber slides were stained with DAPI (Life Technologies, Carlsbad, CA, USA). A microscope (model DM6000B; Leica) equipped with a motorized stage and a digital camera (Olympus, Tokyo, Japan) was used for acquisition of 10 images per chamber. Images were randomly selected by the Visiopharm software (Hoersholm, Denmark) and cell nuclei were counted manually afterwards.

2.3.3 Pepsin digestion, SDS-PAGE and silver stain

Supernatants and cell layers from 12-well-plates were harvested separately and pepsinized as described before [120]. Briefly, 50 µL of a pepsin stock solution (1 mg/ml dissolved in 1N HCL; Roche Applied Sciences, Basel, SUI) were added to 500 µL of supernatants, while a pepsin digestion solution (25% pepsin stock solution, 0.005% Triton X-100 (Bio-Rad Laboratories, Hercules, CA, USA), 0.01% Phenol Red (Sigma-Aldrich St. Louis, MO, USA) in dd H_2O) was added to cell layers. All samples were incubated for two hours on orbital shakers followed by neutralization with 1N NaOH.

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under non-reducing conditions using 3-8% precast Criterion XT Tris-Acetate gels (Bio-Rad Laboratories, Hercules, CA, USA) and 1x XT Tricine running buffer (Bio-Rad Laboratories, Hercules, CA, USA); electrophoresis was performed for 60 minutes at 200V. VitroCol, human collagen I solution (Cell Systems, Troisdorf, Germany) and human collagen solution type III (Cell Systems, Troisdorf, Germany) served as collagen standards (0.16 µg/lane). Gels were subsequently stained with the SilverQuest™ Silver Staining kit (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's protocol. Gel images were acquired using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA), which was also used for densitometric analysis of bands.

2.3.4 Western blot

Proteins were extracted from the cell layer and subjected to SDS-PAGE using Criterion XT Bis Tris Gels (Biorad, Vienna, Austria). Proteins were electroblotted onto Immobilon PVDF membranes (Millipore, MA, US) and immuno-detection was carried out in Tris-buffered saline Tween-20 TBST (Carl Roth, Karlsruhe, Germany) with 2,5% milk or 5% BSA. Membranes were incubated overnight at 4℃ with primary antibodies for detection of fibronectin (FN1; #15613, Proteintech), α-SMA (ACTA2; #LS-B3933, LSBio, WA, US) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; #2118C, Cell Signaling, MA, US). Subsequently, a secondary goat-anti-rabbit (DAKO, Vienna, Austria) or rabbit-anti-goat (DAKO) conjugated to HRP was used for 1h at room temperature. The signal was detected by chemiluminescence.

2.4 Statistical Analysis

Differences of the mean were analyzed by paired t tests after proof of normal distribution using Predictive Analytics Software (PASW) statistics 21.0 (SPSS Inc., Chicago, IL, USA). 0.05 was chosen as level for statistical significance. Normal distribution was given in all parameters, as confirmed by the Kolmogorov-Smirnov test. Biological replicates were treated as independent variables, whereas the technical duplicates were averaged.

3 RESULTS

3.1 MMC in lung fibroblasts

3.1.1 IMR-90

In the first place trials were performed using the IMR-90 cell line, due to its faster growth rates.

Time-dependency is an important factor in ECM production, since especially the extracellular procession of collagen is a tardy procedure in vitro. Moreover media shouldn´t be changed during the trial, in order to not discard unprocessed collagen in the supernatant, which could be incorporated into the ECM during further procession. So there is only a certain amount of FBS available for cells during the whole trial, which will be consumed after a certain time point. The whole experiment was therefore repeated with different culture periods (three, five and ten days) to evaluate the optimal equilibrium between a representative ECM and still healthy, living cells. It revealed that three days was not sufficient for the cells to produce and organize a representative extracellular matrix. ICC pictures showed only little extracellular staining for collagen 1 in either of the aforementioned conditions (data not shown). Moreover ten days were neither suitable since cells did not survive this prolonged culturing period without medium change. In contrast five days did result in a representative amount of extracellular matrix with sufficient staining for the used markers and microscopically healthy cells.

Figures 23 to 26 show ICC pictures of staining in all the used conditions after a culture period of five days. Pictures A in every figure show the staining for collagen 1 at a low magnification (4x) and pictures B at a higher magnification (40x). Pictures C show the DAPI staining for an overview of the distribution of cells as well as the seeding and in pictures D the α-SMA staining in shown.

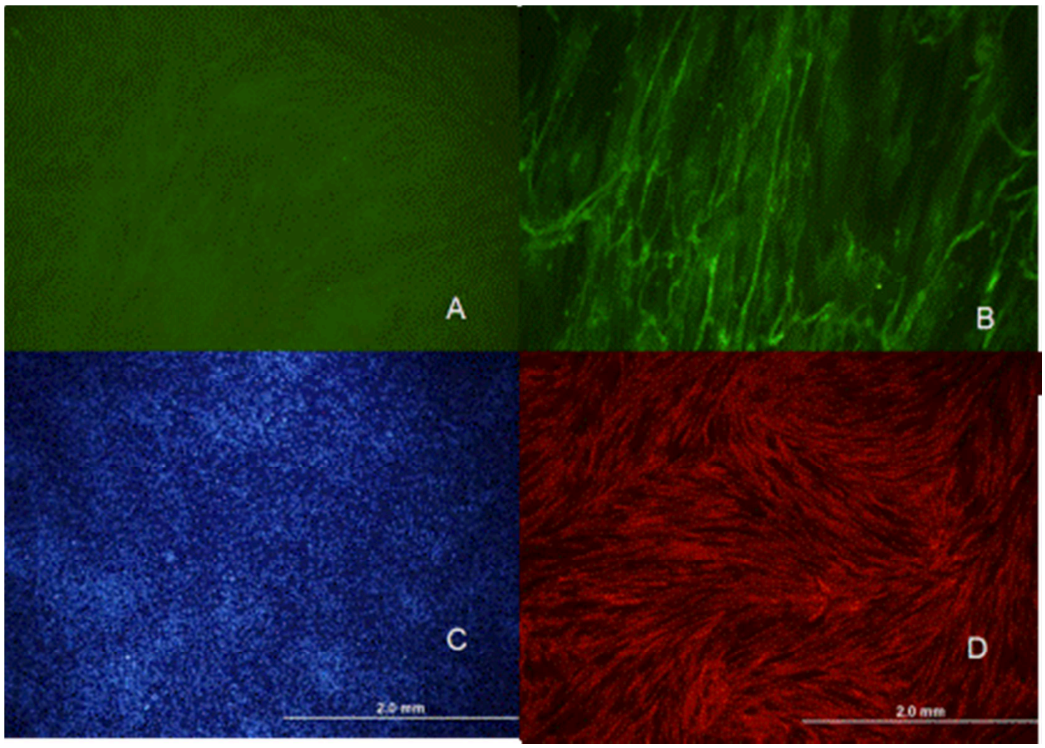

Figure 23: ICC in IMR-90 Control plus standard medium; A:collagen I (4x) B:collagen I (40x) C:DAPI D:α-SMA

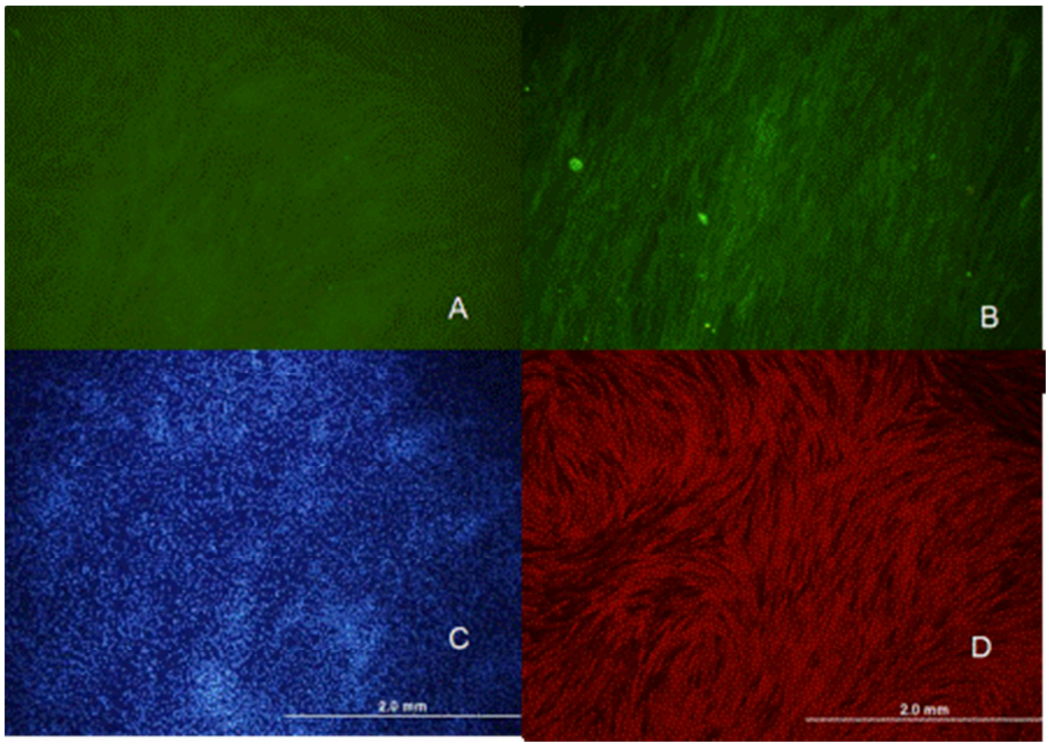

Figure 24: ICC in IMR-90 Control plus crowded medium; A:collagen I (4x) B:collagen I (40x) C:DAPI D:α-SMA

Figure 23 shows the control group with the standard medium, as described above. This was the media, used for most trials with fibroblasts. Picture A in this figure shows a very bright staining for collagen 1 in the 4x magnification. A more detailed description in this view was not possible. But in a higher magnification (40x, picture B) bundles of collagen could be seen, that formed a network in the extracellular space. Noteworthy were the quite big wholes between the bundles in this staining.

Picture C shows an even seeding with regular attachment of cells. In picture D the α-SMA staining is shown, which also gave a quite bright signal. This was actually unexpected, since normal fibroblasts should not express this myofibroblast marker. Note also the difference in the location of the signal between collagen 1 (picture B) and α-SMA (picture D). Collagen 1 is an extracellular signal, whereas α-SMA as part of the cytoskeleton stains intracellularly.

In figure 24, the staining for the crowded group is depicted. In this group a mixture of Fc was added to the standard medium, as described above to enhance the formation of ECM. The low magnification of the collagen 1 staining (picture A) again gave a very bright signal. Compared to picture A of figure 23, the standard medium group without crowding, no big difference can be seen. This changes when we look at the higher magnification (pictures B). In the crowded group (figure 24 picture B) the network of collagen bundles appeared to be more delicate, collagen bundles were also not as thick as in the standard group and the big wholes between the bundles were missing. So the addition of Fc resulted in a more dense formation of extracellular collagen 1.

DAPI staining (picture C) is showing again quite even seeding with no big difference to figure 23.

The expression of α-SMA seemed not to be influenced with crowding, since the staining in picture D is quite similar in figure 23 and 24. This was expected, since crowding influences the ECM procession but should not interfere with the transformation of fibroblasts to myofibroblasts.

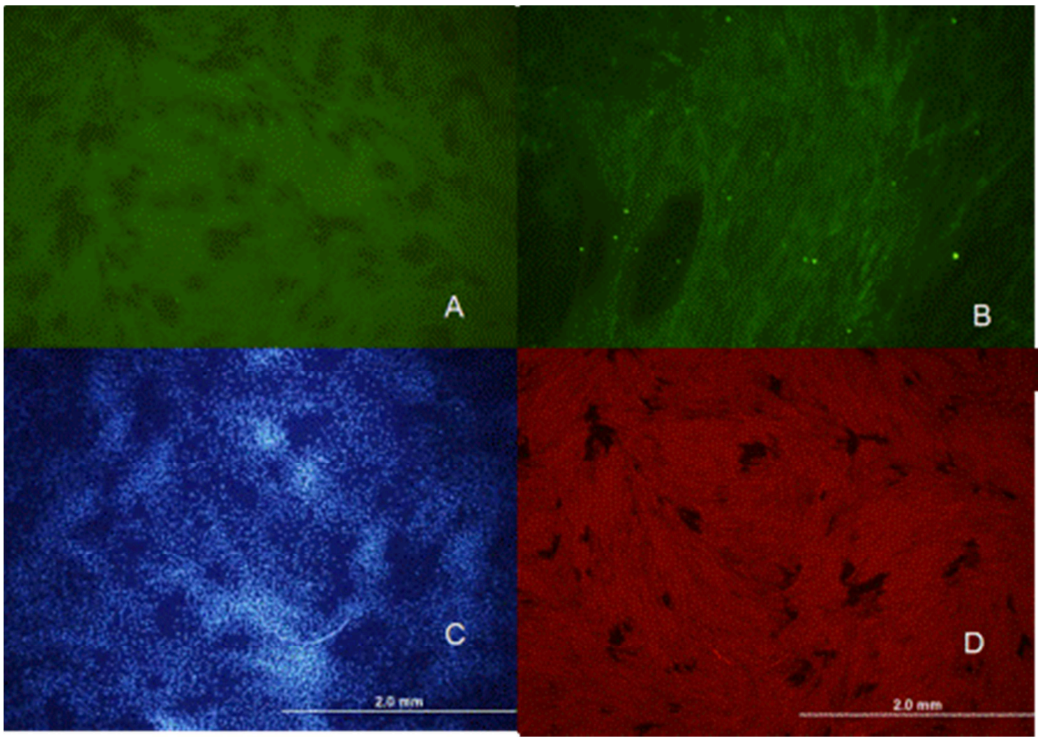

Figure 25: ICC in IMR-90 TGF plus standard medium; A:collagen I (4x) B:collagen I (40x) C:DAPI D:α-SMA

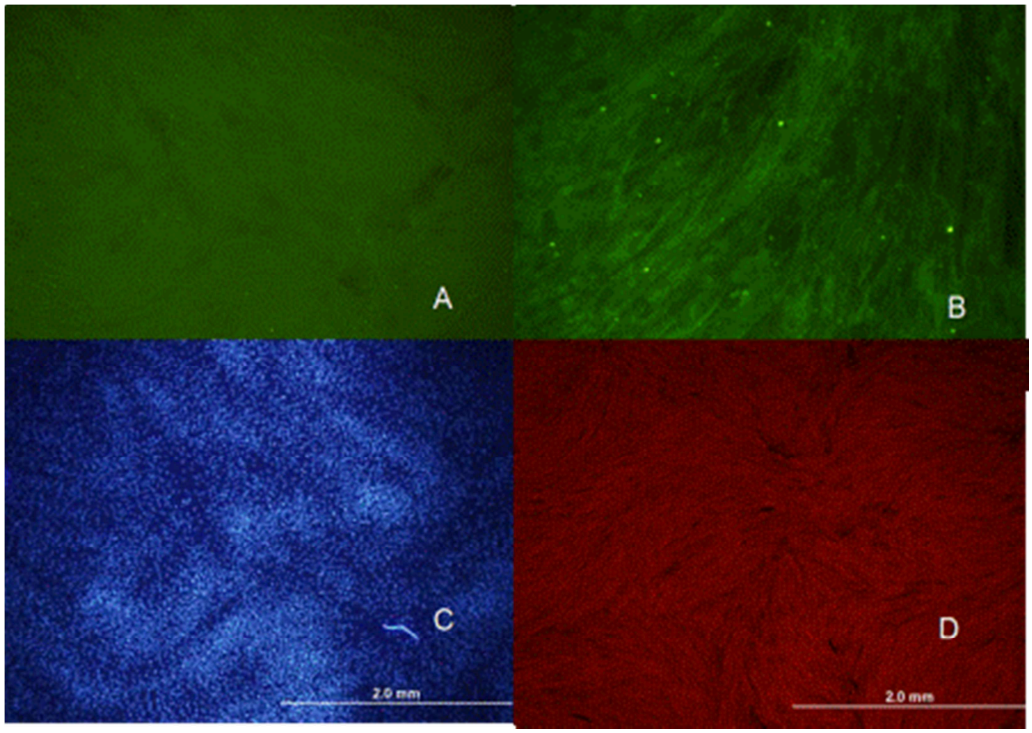

Figure 26: ICC in IMR-90 TGF plus crowded medium; A:collagen I (4x) B:collagen I (40x) C:DAPI D:α-SMA

In the group with standard medium plus TGFβ1 a certain pattern of multiple areas without staining was detected (see figure 25 picture A and B). This was obvious even at low magnifications (picture A). Looking at picture D in this figure reveals also no DAPI staining, meaning no cells in these areas. Uneven seeding could be a reason, but looking at this special pattern, this explanation is unlikely. On the other hand TGFβ1 is a growth factor and therefore could lead to detachment of cells in culture based on its stimulating effects.

In higher magnification (figure 25 picture B) it can be seen that the collagen bundles in this group were quite thin, comparable to the crowded group (figure 24 picture B), but again with quite big holes in between like in the standard group (figure 23 picture B). Note that these holes described in the higher magnification result from collagen procession and are not the same as described above based on cell detachment.

Picture D of figure 25 shows again the α-SMA expression which is dramatically up regulated after TGFβ1 treatment. This indicated the further transformation of fibroblasts to myofibroblasts and is a favoured effect of this growth factor in this setting.

Figure 26 shows the results of the crowded plus TGFβ1 group, representing a “scar-in-a-jar”. In Picture A again a very bright signal for collagen 1 could be seen at low magnification. However ECM free areas, as described above in the setting without Fc, could not be observed. This finding is congruent with the experience, that crowding can extend the attachment period of cells in culture (unpublished data). Picture B shows at a higher magnification once again, that with crowding the collagen 1 bundles were thinner and that big holes in the ECM network, like in standard medium w/o TGFβ1 (pictures B in figure 23 and 25), disappeared.

In picture C the DAPI staining shows in accordance to picture A that cell detachment in the crowded TGFβ1 group was reduced compared to the TGFβ1 group (picture C figure 25).

Finally in picture D of figure 26 a very bright signal for α-SMA could be detected again, which was as mentioned before mainly caused by the effects of TGFβ1.

The aspect of the unexpected high expression of α-SMA of untreated cells, in combination with the aforementioned very high amount of collagen in the ECM in all groups were reasons to repeat the trials with a reduced number of seeded cells per well. The expectation was to get a narrowed signal especially for α-SMA with 20000 instead of 50000, since myofibroblast differentiation could also be induced by the high number of cells per well. Moreover bigger differences in collagen-I-α staining for the different settings were anticipated. However results did just slightly improve with the reduction of cells, especially for α-SMA (figures not shown).

In conclusion for trials with the IMR-90 cell line, TGFβ1 lead to an upregulation of α-SMA, the myofibroblast marker, and of collagen 1, but with insufficient incorporation into the ECM (big holes in the network). Crowding resulted in a more dense and delicate extracellular network of collagen 1 and did not have relevant effects on α-SMA. Even so to generate a representative in-vitro model for scarring both attempts seem to be necessary.

3.1.2 WI-38

The same trial was performed for the WI-38 cell line. Concerning the culturing periods results were similar to the IMR-90 cells with an immature matrix at three days and cells dying around day eight with no usable results for day ten. Further trials were performed with five days of culturing period.

Moreover for ICC the antibody for collagen 1 was changed because of the high background signal (see figures 24-26/ pictures A and B). The newly used primary antibody was rabbit anti human collagen 1, with the disadvantage that we could not stain for α-SMA in the same well because the only available primary antibody against α-SMA was also produced in rabbit. Figures 27 to 30 show the pictures of the collagen staining.

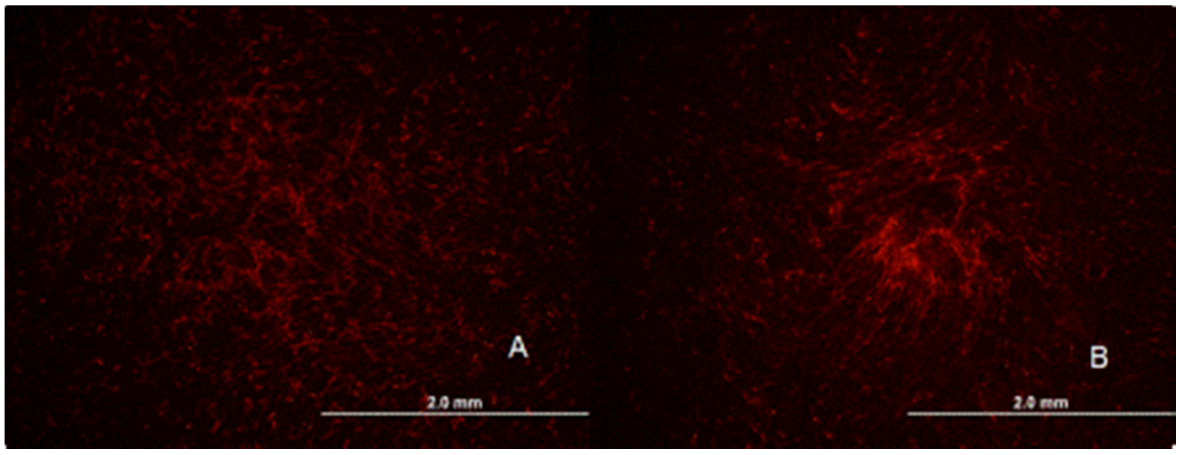

Figure 27: ICC for collagen 1 in WI-38 control groups; A:standard B:crowded medium

Figure 27 shows the collagen 1 staining in the control group with conventional culture conditions as described above (picture A) and after adding a mixture of Fc as inert macromolecules (picture B). Cells seemed to lay down collagen 1 in both conditions, whereas the ECM network was denser in picture B, with crowding, especially at the centre of the well. Moreover as described above, ECM free areas appeared in higher frequency in standard culture conditions and decreased through crowding.

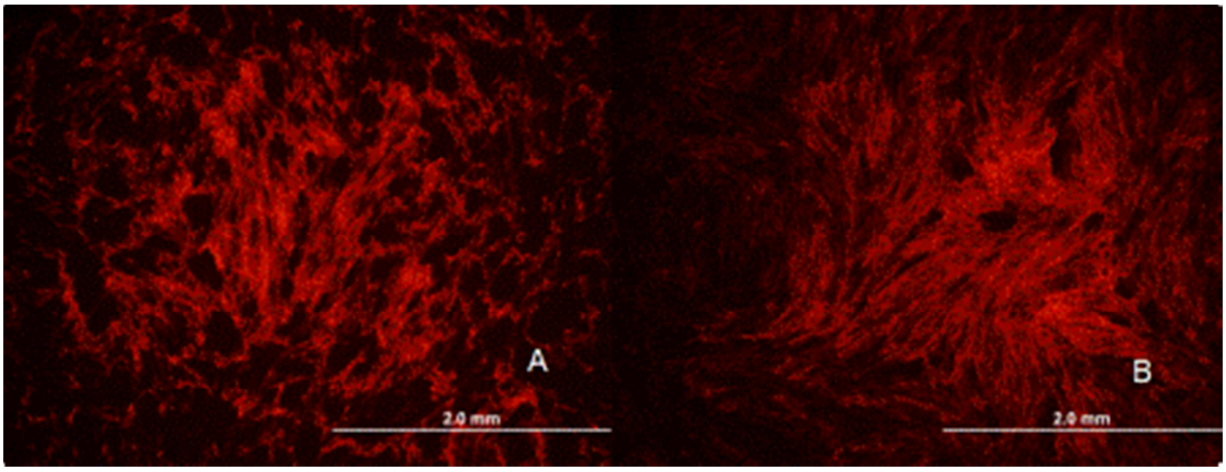

Figure 28: ICC for collagen 1 in WI-38 TGF groups; A: standard B: crowded medium

In figure 28, results after adding TGFβ1 in standard (picture A) and in crowded medium (picture B) are shown. Comparing picture A of figure 28 and 27 reveals again up regulated collagen 1 after adding TGFβ1 to standard medium, with a brighter staining in figure 28. At the same time ECM free areas seemed to increase, most likely because of cell detachment after growth stimulation by TGFβ1, as described above.

Adding macromolecules and TGFβ1 resulted in an even more increased collagen 1 deposition (figure 28 picture B) than adding TGFβ1 alone (picture A). In contrast collagen-free areas were again reduced by adding macromolecules. This condition with TGFβ1 plus macromolecules enabled the highest amount of in-vitro produced ECM, representing fibrosis.

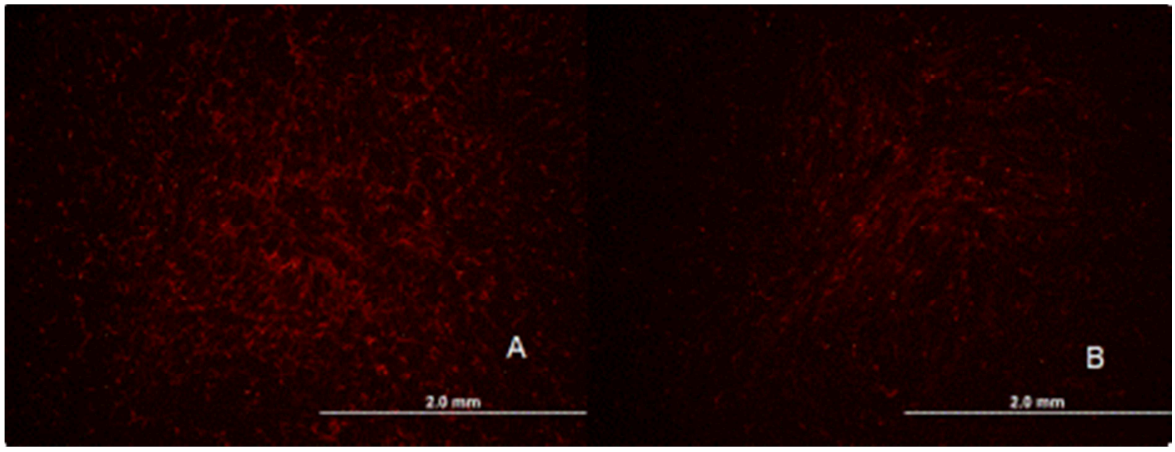

Figure 29: ICC for collagen 1 in WI-38 HGF groups ; A:standard B:crowded medium

Figure 29 shows the collagen deposition in the HGF treated groups with standard (picture A) and crowded medium (picture B). Herein it was remarkable that collagen 1 staining was brighter in picture A than in picture B. Meaning that collagen 1 in the ECM was decreased more efficiently under crowded conditions in comparison to standard medium.

The reduction of collagen 1 under the influence of HGF is also obvious when we compare picture B of figure 29 and picture B of figure 27, showing that the increase in collagen 1 resulting from crowding effects could be influenced by growth factors. In contrast, comparing pictures A of figures 29 and 27 did not show such an obvious effect of HGF when added to standard medium.

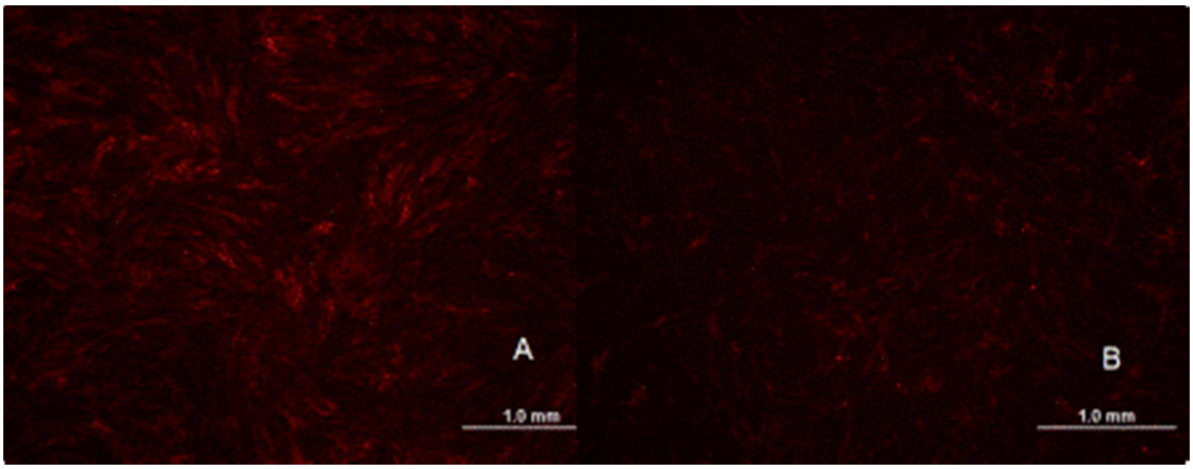

Figure 30: ICC for collagen 1 in WI-38 w/o HGF; A:TGF crowded medium B:TGF HGF crowded medium

Finally in figure 30 the combined condition of crowding plus TGFβ1 alone (picture A) is compared to the combined condition with additionally HGF (picture B). A decreased staining for collagen 1 in picture B was obvious.

So crowding did not only enhance ECM formation especially tested at the level of collagen 1, but also allowed for in vitro testing of antifibrotic agents. With these encouraging results tests were shifted to the level of vocal folds.

3.2 MMC in pVFF

3.2.1 Collagen

	Collagen Iα [ng/ml]		
	non-crowded	crowded	p-value[B]
supernatant			
control	379.5±23.8	3152.8±94.6	≤0.05
TGF	3382.8±122.5	3855.5±226.9	0.099
p-value[A]	≤0.05	0.199	
cell layer			
control	336.1±55.0	1251.8±142.9	≤0.05
TGF	709.8±136.4	1303.0±136.2	≤0.05
p-value[A]	≤0.05	0.839	

Table 3: Results from ELISA for collagen in pVFF in different conditions; p-value[A] comparing control and TGF; p-value[B] comparing uncrowded and crowded; TGF=transforming growth factor.

Results from ELISA for collagen 1 in different conditions are shown in table 3. Deposition was significantly enhanced under crowded conditions compared to standard culture medium in supernatant (379.5±23.8 ng/ml vs. 3152.8±94.6 ng/ml; p≤0.05) as well as in the cell layer (336.1±55.0 ng/ml vs. 1251.8±142.9 ng/ml; p≤0.05). The administration of TGF-β alone significantly increased the deposition of collagen 1 in the supernatant (379.5±23.8 ng/ml vs. 3382.8±122.5 ng/ml; p≤0.05) and in the cell layer (336.1±55.0 ng/ml vs. 709.8±136.4 ng/ml; p≤0.05) compared to control. The combination of crowding and TGF-β also significantly increased collagen 1 deposition in the supernatant and in the cell layer compared to the uncrowded control (379.5±23.8 ng/ml vs. 3855.5±226.9 ng/ml; p≤0.05; resp. 336.1±55.0 ng/ml vs. 1303.0±136.2 ng/ml; p≤0.05)(see also figure 32). Moreover, the combination of crowding and TGF-β administration resulted in a significant increase of collagen 1 levels in the cell layer compared to TGF-β administration alone (1303.0±136.2 ng/ml vs. 709.8±136.4 ng/ml; p≤0.05) but not when compared to crowding alone (1303.0±136.2 ng/ml vs. 1251.8±142.9 ng/ml; p=0.839). In the supernatant the

combination of TGF-β and macromolecules did not significantly increase collagen 1 content over TGF-β or Ficoll alone (3855.5±226.9ng/ml vs. 3382.8±122.5ng/ml; p=0.099; resp. vs. 3152.8±94.6ng/ml; p=0.199).

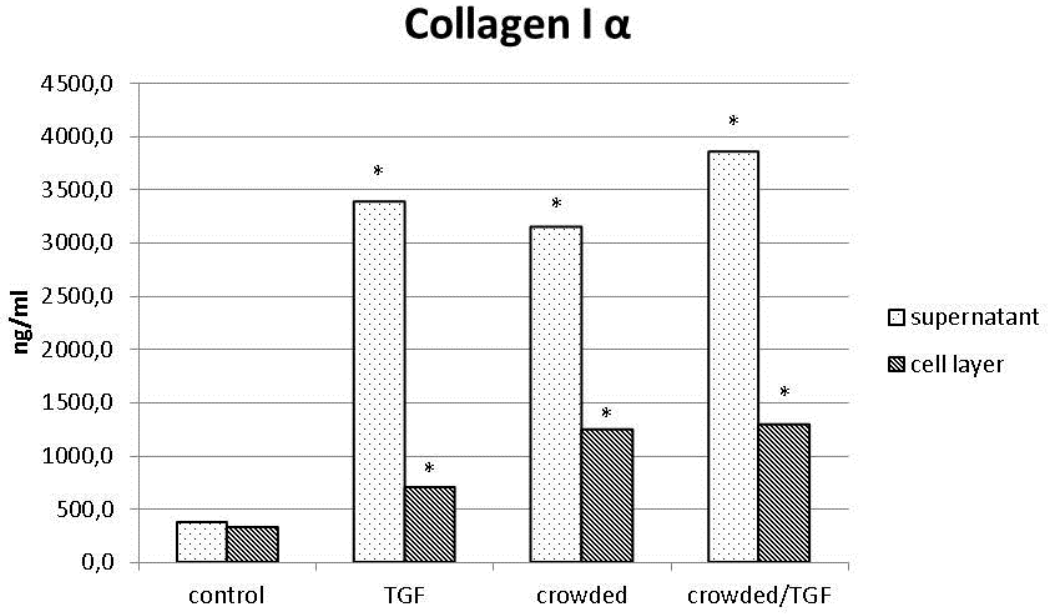

Figure 31: Deposition of collagen 1 in pVFF in different conditions. * p≤.05 versus control in supernatant resp. cell layer

Figure 32 shows even clearer the big difference in collagen 1 content in supernatant and cell layer between conventional culture conditions of fibroblasts and groups after treatment with TGF-β and/or macromolecules. Note especially the difference in cell layer between the group with TGF-β and the group with crowding alone. Crowding results in nearly a doubling of collagen-I-α in cell layer compared to TGF-β alone (1251.8±142.9 ng/ml vs. 709.8±136.4 ng/ml), whereas in supernatant collagen 1 was slightly higher in the TGF-β group than in the group with ficoll alone (3382.8±122.5ng/ml vs. 3152.8±94.6ng/ml).

Results of immunocytochemistry correlated with results achieved from ELISA trials and are shown in figure 33. Deposition of collagen 1 in the group with standard medium was sparse, showing only minimal extracellular signal (see upper picture in figure 33). Addition of macromolecules alone dramatically increased staining for collagen 1 with a very dense signal in nearly the whole area of the chamber (see middle picture in figure 33). The lower picture of figure 33 shows ICC for the group after combined treatment with macromolecules and TGF-β. Staining in these chambers was even brighter than with macromolecules alone with several areas of maximal signal.

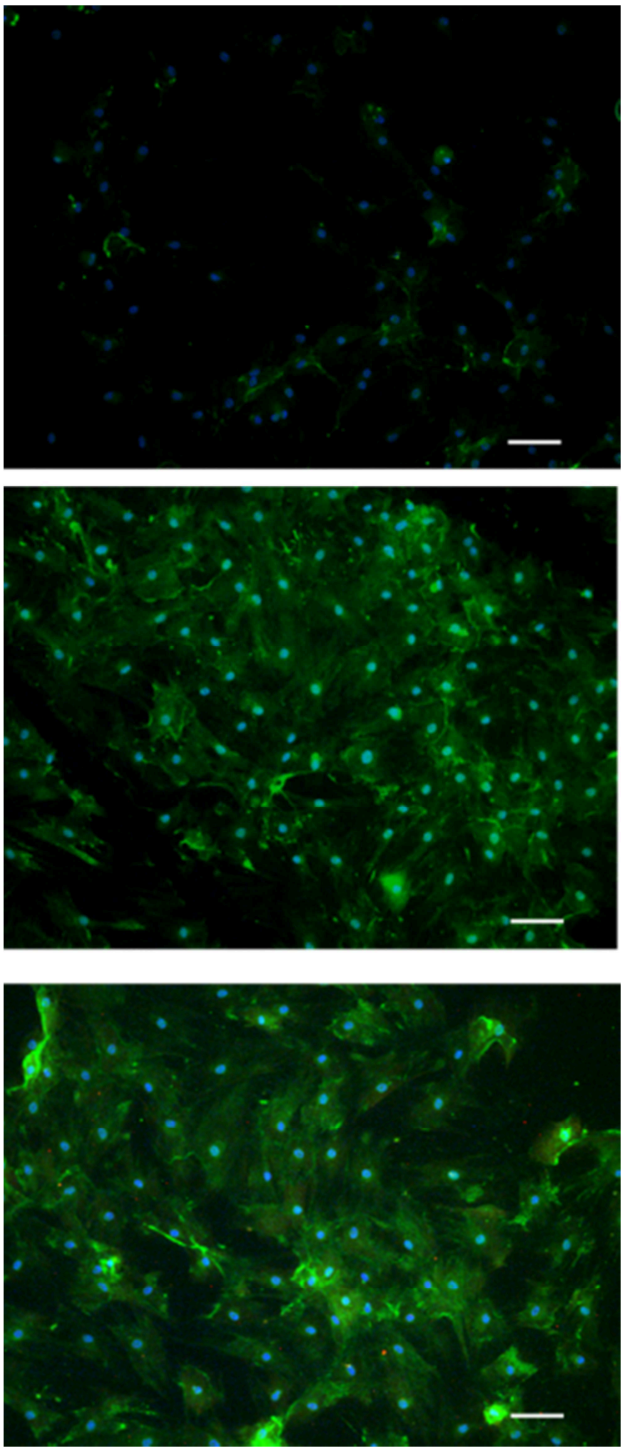

Figure 32: ICC of pVFF for collagen 1; Each row represents different conditions: upper: non-crowded control middle: crowded control; lower: crowded TGF-β; Scale bar: 100µm

3.2.2 Hyaluronic acid (HA)

Hyaluronic acid [ng/ml]			
	non-crowded	crowded	p-value[B]
supernatant			
control	429.8±25.5	1053.3±31.9	≤0.05
TGF	1064.8±31.9	1295.8±58.7	≤0.05
p-value[A]	≤0.05	≤0.05	
cell layer			
control	180.0±20.5	642.5±73.1	≤0.05
TGF	323.0±45.0	712.0±67.2	≤0.05
p-value[A]	≤0.05	0.124	

Table 4: Results from ELISA in pVFF for hyaluronic acid in different conditions; p-value[A] comparing control and TGF; p-value[B] comparing uncrowded and crowded; TGF=transforming growth factor

Hyaluronic acid content in supernatant and cell layer in groups with different conditions is shown in Table 4. Crowding led to significantly increased levels of HA in supernatants (429.8±25.5ng/ml vs. 1053.3±31.9ng/ml, p≤0.05) as well as in the cell layer (180.0±20.5ng/ml vs. 642.5±73.1ng/ml, p≤0.05), when compared to non-crowded control. Administration of TGF-β also enhanced the amount of HA significantly compared to control under non-crowded conditions in supernatant (429.8±25.5ng/ml vs. 1064.8±31.9ng/ml, p≤0.05) and in cell layer (180.0±20.5ng/ml vs. 323.0±45.0ng/ml, p≤0.05). Moreover administering ficoll additionally to TGF-β resulted in a further upregulation of HA in both compartments the supernatant (1295.8±58.7ng/ml vs. 1064.8±31.9ng/ml, p≤0.05) and the cell layer (712.0±67.2ng/ml vs. 323.0±45.0ng/ml, p≤0.05) compared to TGF-β alone. In contrast, when comparing the crowded group with the group of ficoll plus TGF-β we found that HA was significantly increased in supernatant after combined treatment (1295.8±58.7ng/ml vs. 1053.3±31.9ng/ml, p≤0.05), but not in cell layer (712.0±67.2ng/ml vs.

642.5±73.1ng/ml, p=0.124). So TGF-β did not seem to have significant additionally effects to crowding in cell layer.

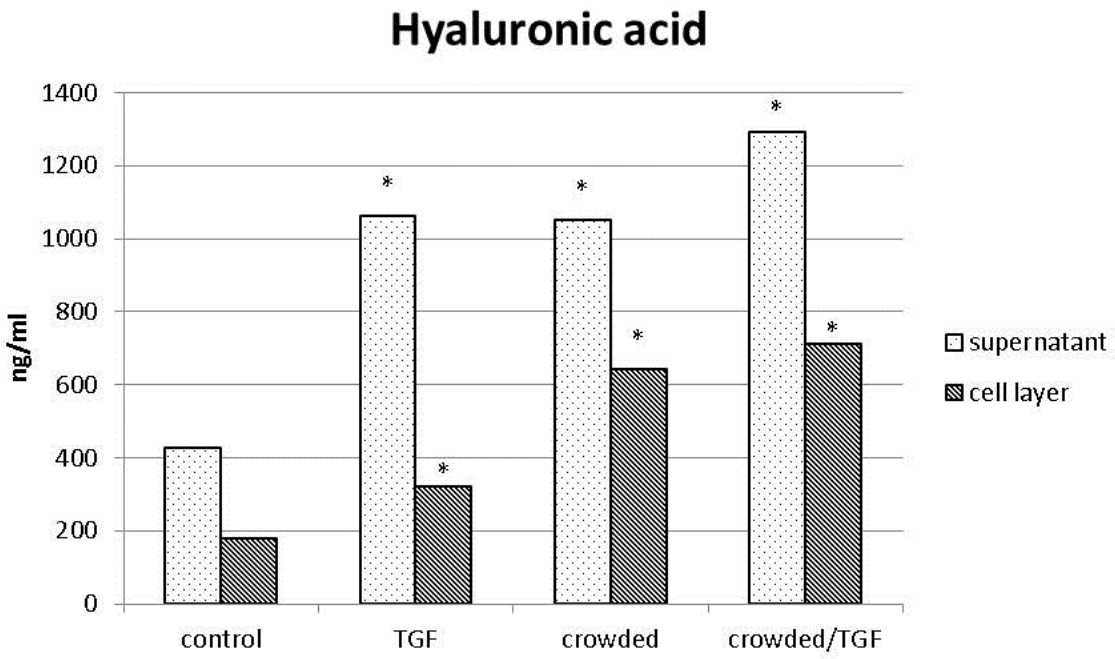

Figure 33: Deposition of Hyaluronic acid by pVFF in different conditions. * p≤.05 versus control in supernatant resp. cell layer

In addition, the combination of crowding and TGF-β highly increased HA in supernatant (429.8±25.5ng/ml vs. 1295.8±34.6ng/ml, p≤0.05) and cell layer (180.0±20.5ng/ml vs. 712.0±67.2ng/ml, p≤0.05) compared to the untreated control groups with standard cell culture medium (see figure 35). The difference was especially obvious in the cell layer with a nearly four-fold increase. Moreover it can be seen clearly in figure 35 that ficoll seems to have a more pronounced effect in the compartment of the cell layer than TGF-β.

3.2.3 Fibronectin

Fibronectin [µg/ml]			
	non-crowded	crowded	p-value[B]
supernatant			
control	9.2±0.9	13.1±1.8	0.124
TGF	15.8±1.6	28.3±3.2	≤0.05
p-value[A]	≤0.05	≤0.05	
cell layer			
control	0.5±0.04	1.3±0.1	≤0.05
TGF	0.7±0.03	1.7±0.04	≤0.05
p-value[A]	≤0.05	≤0.05	

Table 5: Results from ELISA for fibronectinin pVFF in different conditions; p-value[A] comparing control and TGF; p-value[B] comparing uncrowded and crowded; TGF=transforming growth factor

Results for fibronectin level in different compartments after different treatment of cells are shown in table 5. Crowding was able to increase fibronectin significantly in cell layer compared to standard culture conditions (0.5±0.04µg/ml vs. 1.3±0.1µg/ml, p≤0.05), whereas in supernatant results were not significantly different (9.2±0.9µg/ml vs. 13.1±1.8µg/ml, p=0.124), showing again the stronger effect of ficoll within the cell layer. Furthermore TGF-β was also able to up regulate fibronectin compared to control in supernatant (9.2±0.9µg/ml vs. 15.8±1.6µg/ml, p≤0.05) and in cell layer (0.5±0.04µg/ml vs. 0.7±0.03µg/ml, p≤0.05). The combination of the used agents led moreover to a further significant increase of fibronectin content in the cell layer compared to either ficoll (1.7±0.04µg/ml vs. 1.3±0.1µg/ml, p≤0.05) or TGF-β (1.7±0.04µg/ml vs. 0.7±0.03µg/ml, p≤0.05) alone. Similar results were also seen in supernatants (28.3±3.2µg/ml vs. 13.1±1.8µg/ml, p≤0.05; resp. vs 15.8±1.6µg/ml, p≤0.05). The upregulation of fibronectin after combined treatment of cells was even more obvious when comparing to results from cells after standard culture conditions (28.3±3.2µg/ml vs. 9.2±0.9µg/ml, p≤0.05; resp. 1.7±0.04 vs 0.5±0.04µg/ml, p≤0.05) (see also figure 37).

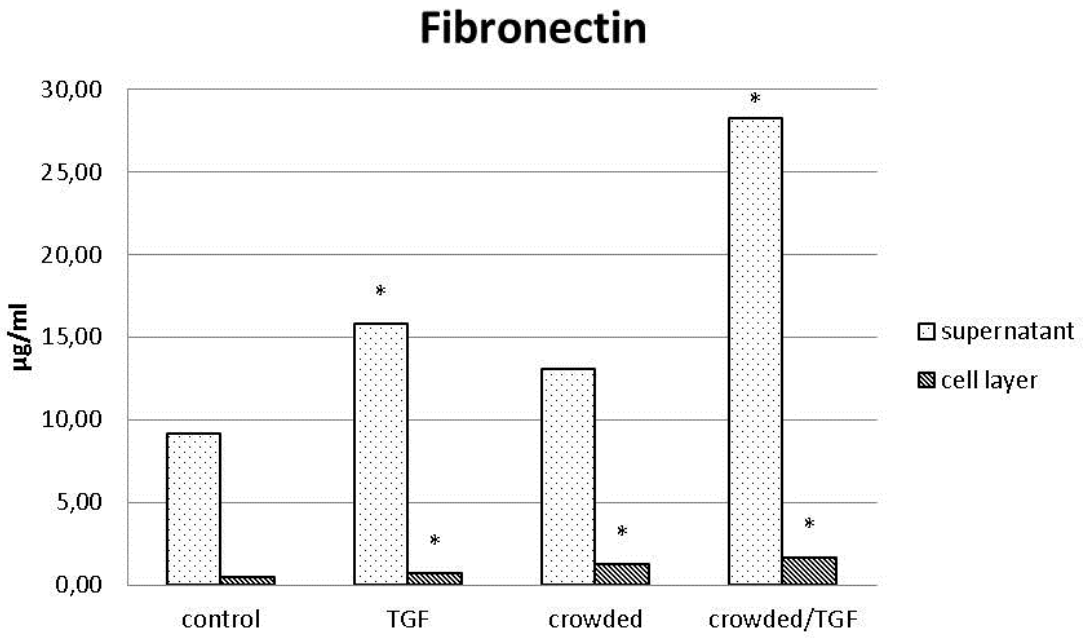

Figure 34: Deposition of Fibronectin by pVFF in different conditions. * p≤.05 versus control in supernatant resp. cell layer

3.2.4 α-SMA

α-SMA [ng/ml]			
	non-crowded	crowded	p-value[B]
cell layer			
control	b.r.	9.33±3.8	-
TGF	7.7±0.6	10.0±1.7	0.118
p-value[A]	-	0.774	

Table 6: Results from ELISA for α-SMA in pVFF in different conditions; p-value[A] comparing control and TGF; p-value[B] comparing uncrowded and crowded; TGF=transforming growth factor

As described above α-SMA was determined only from the cell layer after lysing, as it is part of the cytoskeleton. See table 6 for results from ELISA. In non-crowded control samples α-SMA was below range, indicating their non-scar condition and expression patterns. Therefore, this group had to be excluded from statistical analysis. In the other groups α-SMA was detectable, with the lowest value after administration of TGF-β (7.7±0.6ng/ml). Crowding alone resulted in slightly elevated values (9.33±3.8ng/ml), as well as the combination of crowding and TGF-β (10.0±1.7ng/ml). However there was no statistically significant difference between the groups (see also figure 39).

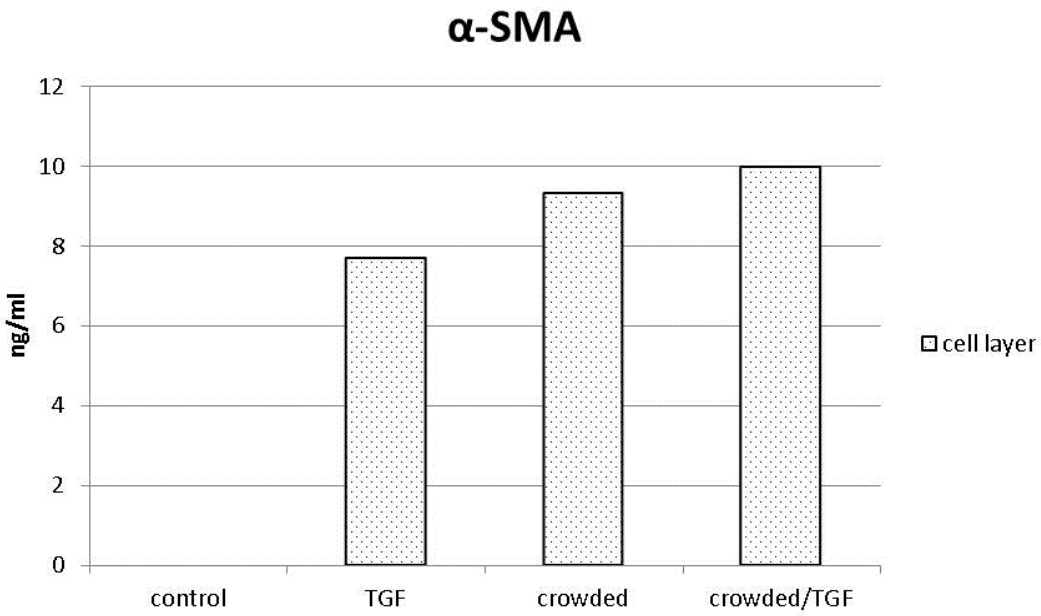

Figure 35: Deposition of α-SMA by pVFF in different conditions

3.2.5 HGF

As described above HGF was administered at a concentration of 40ng/ml in one group additionally to combined treatment with ficoll and TGF-β to test for antifibrotic properties. This resulted in a slight decrease of collagen compared to the group without HGF in cell layer (692.8±26.9ng/ml vs. 658.3±81.1ng/ml). In supernatant HGF did interestingly induce an increase in collagen compared to the untreated group (578.7±104.2ng/ml vs. 665.5±45.5ng/ml). However, these results did not reach statistical significance (see figure 40). Since HGF as a growth factor is also able to induce cell growth, an increase in cell number, each cell producing collagen, could be a possible explanation for the higher collagen content in supernatant after HGF treatment. However this was not further investigated in this setting.

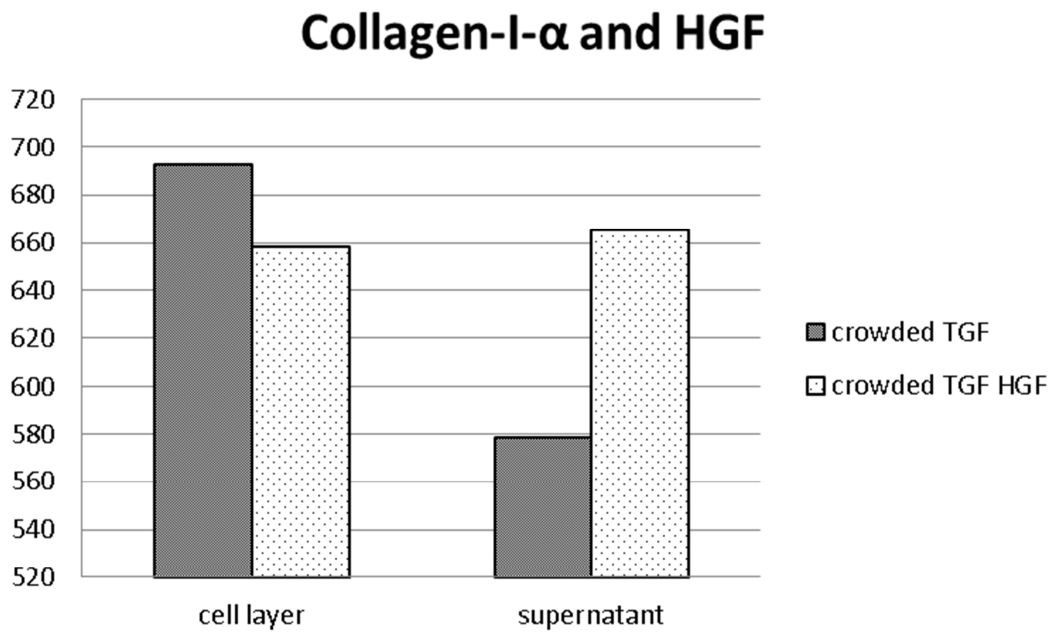

Figure 36: Effect of HGF on collagen 1 in pVFF

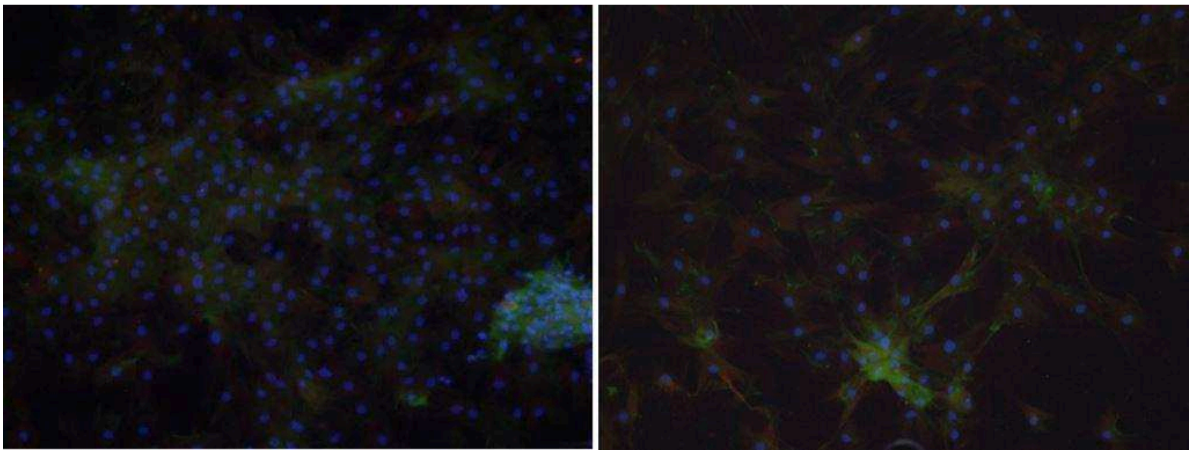

Figure 37: ICC for collagen 1 in pVFF w/o HGF; left picture: crowding + TGF-β; right picture: crowding + TGF-β+ HGF

In immunofluorescence staining collagen 1 was also slightly decreased after adding HGF with a lower signal for collagen in the HGF treated group (see figure 41).

3.3 MMC in hVFF

3.3.1 Collagen

Silver stain results for collagen deposition under different conditions are shown in figure 42A. In the non-crowded control group (figure 42A: NC/C) collagen 1 was detected in supernatant, but hardly in cell layer. After adding TGFβ-1 (figure 42A: NC/T) the signal increased in supernatant as well as in the cell layer, whereas crowding alone (figure 42A: C/C) resulted in an increased signal only in cell layer, but not in supernatant. Finally the combination of TGFβ-1 and crowding (figure 42A: C/T) led to a pronounced deposition of collagen 1 in cell layer, with only residual bands in supernatant. Densitometric analysis showed corresponding results (see figure 42B). Mark especially again that adding ficoll (figure 42B: C/C) resulted in a decrease of collagen in supernatant and an increase in cell layer compared to standard medium (figure 42B: NC/C). TGF alone increased collagen as described above in both compartments (figure 42B: NC/T) and the combination of both agents (figure 42B:

C/T) increased collagen over the level of every single agent in cell layer, but in supernatant collagen content was decreased after combined treatment especially when compared to TGF-β alone.

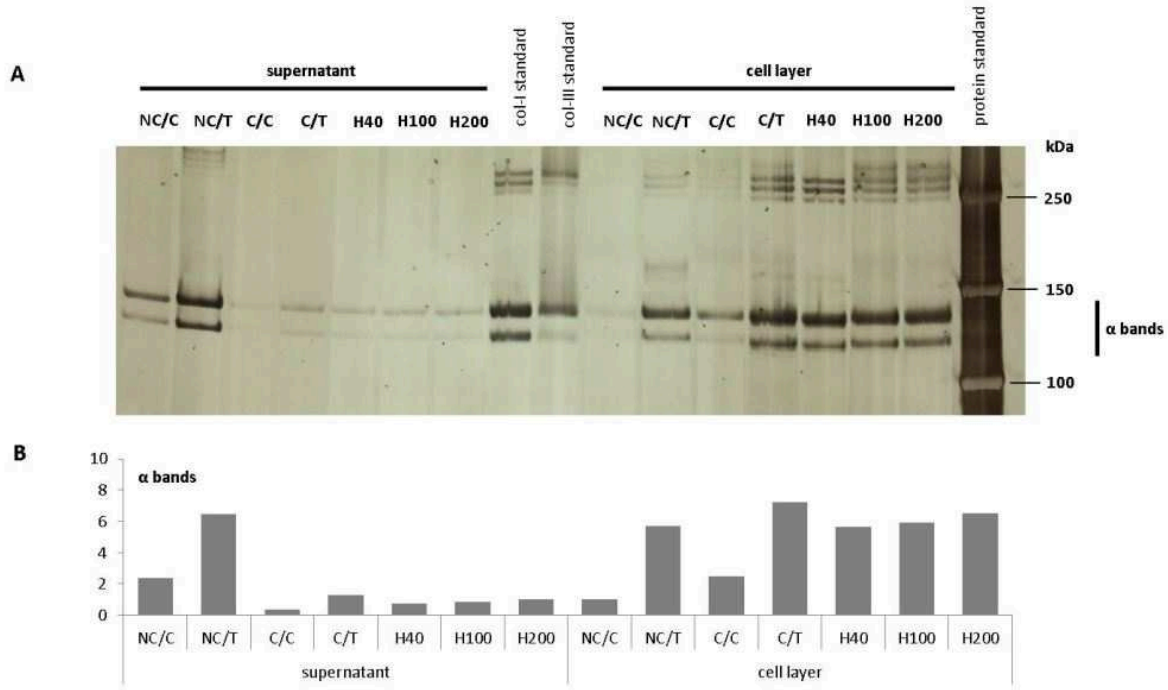

Figure 38: A Silver stain results in hVFF for collagen deposition in standard conditions and with HGF treatment; NC/C=non-crowded control; C/C=crowded control; NC/T=non-crowded+TGF-β1; C/T=crowded+TGF-β1; H40=crowded+TGF-β1+HGF40ng/ml; H100=crowded+TGF-β1+HGF100ng/ml; H200ng/ml; B Densitometric analysis of silver stain for collagen deposition in hVFF; condition NC/C of cell layer served as reference

Similar results were observed in immunocytochemistry (figure 43 a-d) with a dramatically enhanced signal when combining crowding and TGFβ-1 (figure 43: d) compared to either of the two components alone (figure 43: b and c), or to standard medium (figure 43: a).

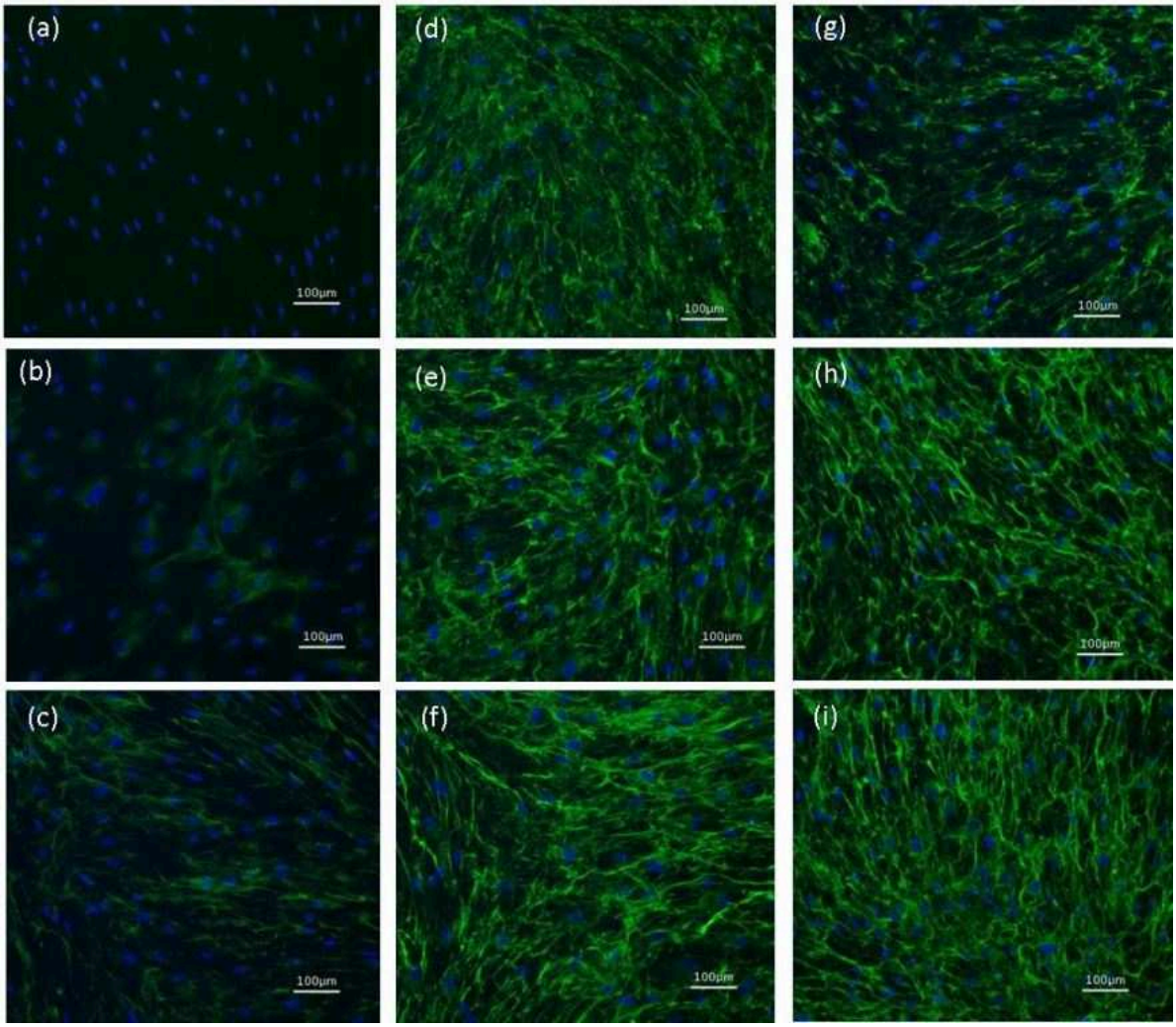

Figure 39: ICC of hVFF for collagen I in standard conditions and after treatment with Botox or HGF; (a) non-crowded control; (b) non-crowded+TGF-β1; (c) crowded control; (d) crowded+TGF-β1; (e) crowded+TGF-β1+Botox40IU/ml; (f) crowded+TGF-β1+Botox 80IU/ml

Addition of HGF at different concentrations showed a decrease in silver staining for α-bands in cell layer at 40ng/ml HGF (figure 42A: H40) compared to the crowded plus TGFβ-1 group (figure 42A: C/T). With an increase in concentration of HGF this effect could not be seen any more (figure 42A: H100 and H200). Densitometric analysis of bands is shown in figure 42B. Similarly to silver staining, the collagen signal in immunocytochemistry decreased when 40ng/ml HGF was added (figure 43: g vs. d), but increased again at concentrations of 100 and 200ng/ml of HGF (figure 43: h and

i). To further investigate a potential enhanced cell growth effect during increasing HGF concentrations -which might counteract the antifibrotic properties- cells were counted as described above, revealing a significant boost in cell numbers in chambers with high HGF concentrations (see figure 44).

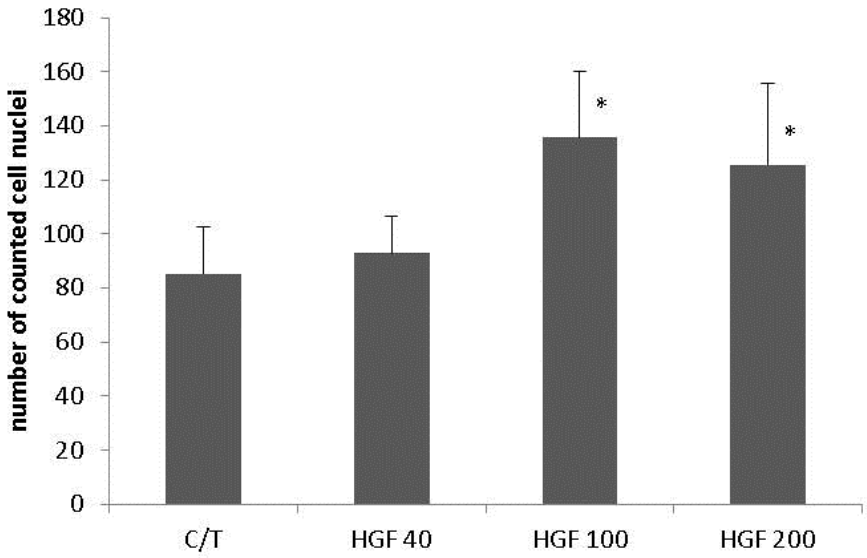

Figure 40: Cell count of hVFF after HGF treatment; C/T=crowded+TGF-β1; H40=crowded+TGF-β1+HGF40ng/ml; H100=crowded+TGF-β1+HGF100ng/ml; H200=crowded+TGF-β1+HGF 200ng/ml; * p<0.05 vs. C/T

Botox was also tested in two different concentrations (40IU/ml or 80IU/ml) for potential antifibrotic properties, but did not show an effect on collagen neither in silver staining (figure 45) nor in immunocytochemistry (figure 43: e and f).

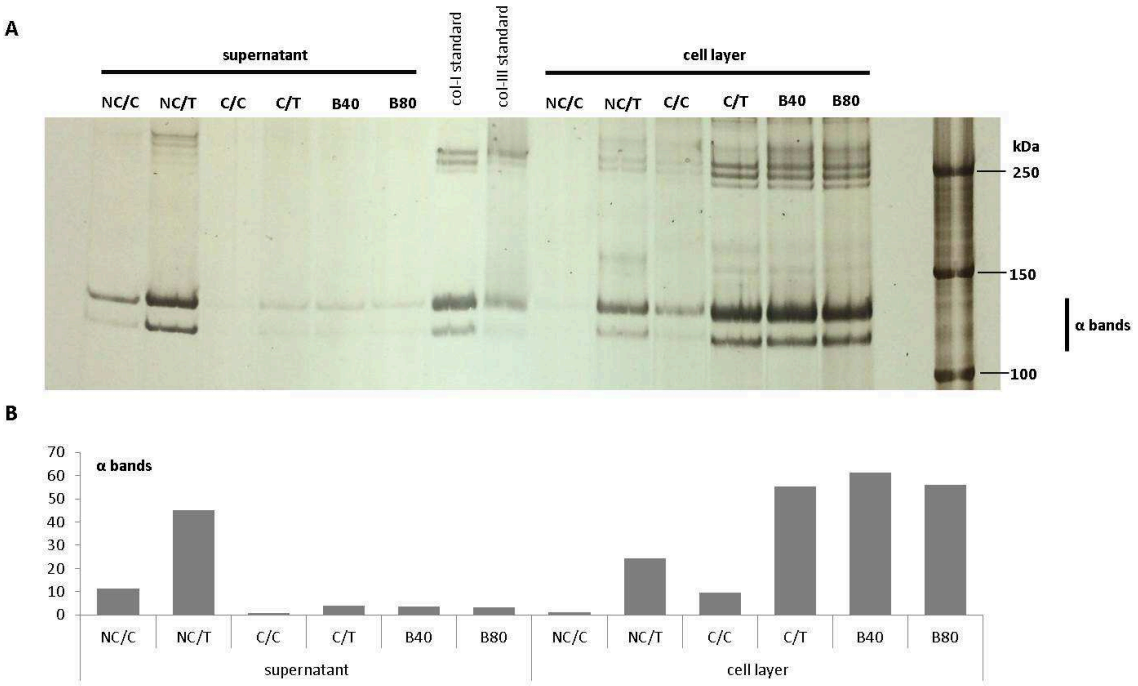

Figure41: A Silver stain results of hVFF for collagen deposition in standard conditions and with Botox treatment; NC/C=non-crowded control; C/C=crowded control; NC/T=non-crowded+TGF-β1; C/T=crowded+TGF-β1; B40=crowded+TGF-β1+Botox40IU/ml; B80=crowded+TGF-β1+Botox80IU/ml; **B** Densitometric analysis of silver stain for collagen deposition; condition NC/C of cell layer served as reference

3.3.2 Fibronectin

Results for Western blot are shown in figure 46A. Addition of TGFβ-1 (figure 46A: NC/T) or crowding conditions (figure 46A: C/C) enhanced separately fibronectin deposition in cell layer, whereas the combination of both (figure 46A: C/T) resulted in a more pronounced upregulation exceeding a simple summation of single effects. Adding HGF resulted in a decline of fibronectin depending on the used concentration with the most prominent effects at 40ng/ml similar to the results for collagen. Furthermore Botox did not show strong effects. These results were corroborated by immunocytochemistry (figure 46B).

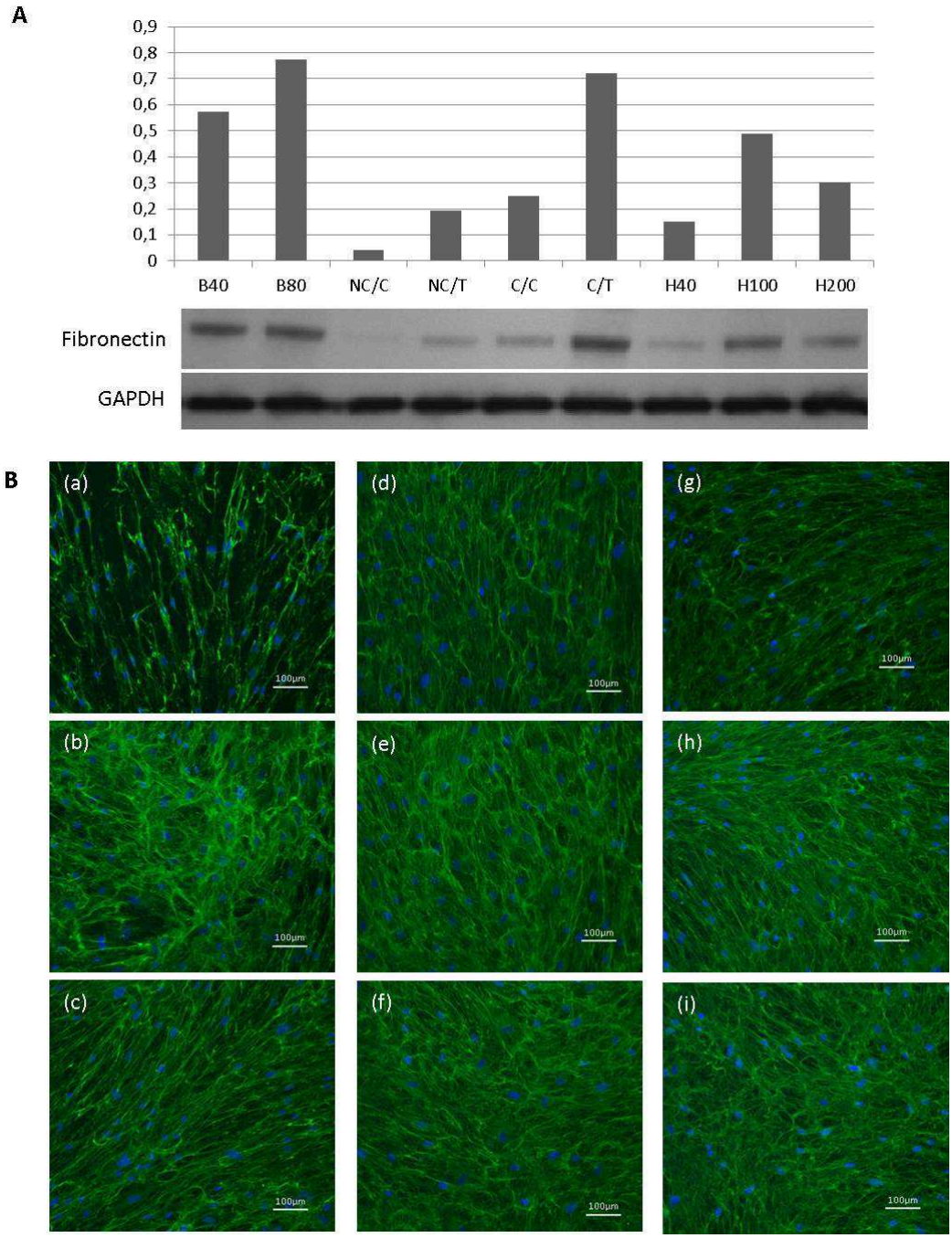

Figure 42: A Westernblot for fibronectin in hVFF; NC/C=non-crowded control; C/C=crowded control; NC/T=non-crowded+TGF-β1; C/T=crowded+TGF-β1; H40=crowded+TGF-β1+HGF40ng/ml; H100=crowded+TGF-β1+HGF100ng/ml; H200=crowded+TGF-β1+HGF200ng/ml; B40=crowded+TGF-β1+Botox40IU/ml; B80=crowded+TGF-β1+Botox80IU/ml; **B** Immunocytochemistry for fibronectin in standard conditions and after treatment with Botox and HGF; (a) non-crowded control; (b) non-crowded+TGF-β1; (c) crowded control; (d) crowded+TGF-β1; (e) crowded+TGF-β1+Botox40IU/ml; (f) crowded+TGF-β1+Botox80IU/ml; (g) crowded+TGF-β1+HGF40ng/ml; (h) crowded+TGF-β1+HGF100ng/ml; (i) crowded+TGF-β1+HGF200ng/ml;

3.3.3 α-SMA

Signals for α-SMA could not be detected in densitometric analyses of Western blot within groups cultured with standard medium (figure 47A: NC/C), or crowding alone (figure 47A: C/C). Treating cells with TGFβ-1 induced high expression of α-SMA in the absence and also presence of crowding molecules (figure 47A: NC/T and C/T). HGF was able to reverse this effect in a dose dependent manner with decreasing α-SMA signals at increasing HGF concentrations (figure 47A: H40, H100 and H200). The addition of Botox was not effective in suppressing α-SMA. For both HGF and Botox, Immunocytochemistry analysis of α-SMA showed corresponding results (figure 47B).

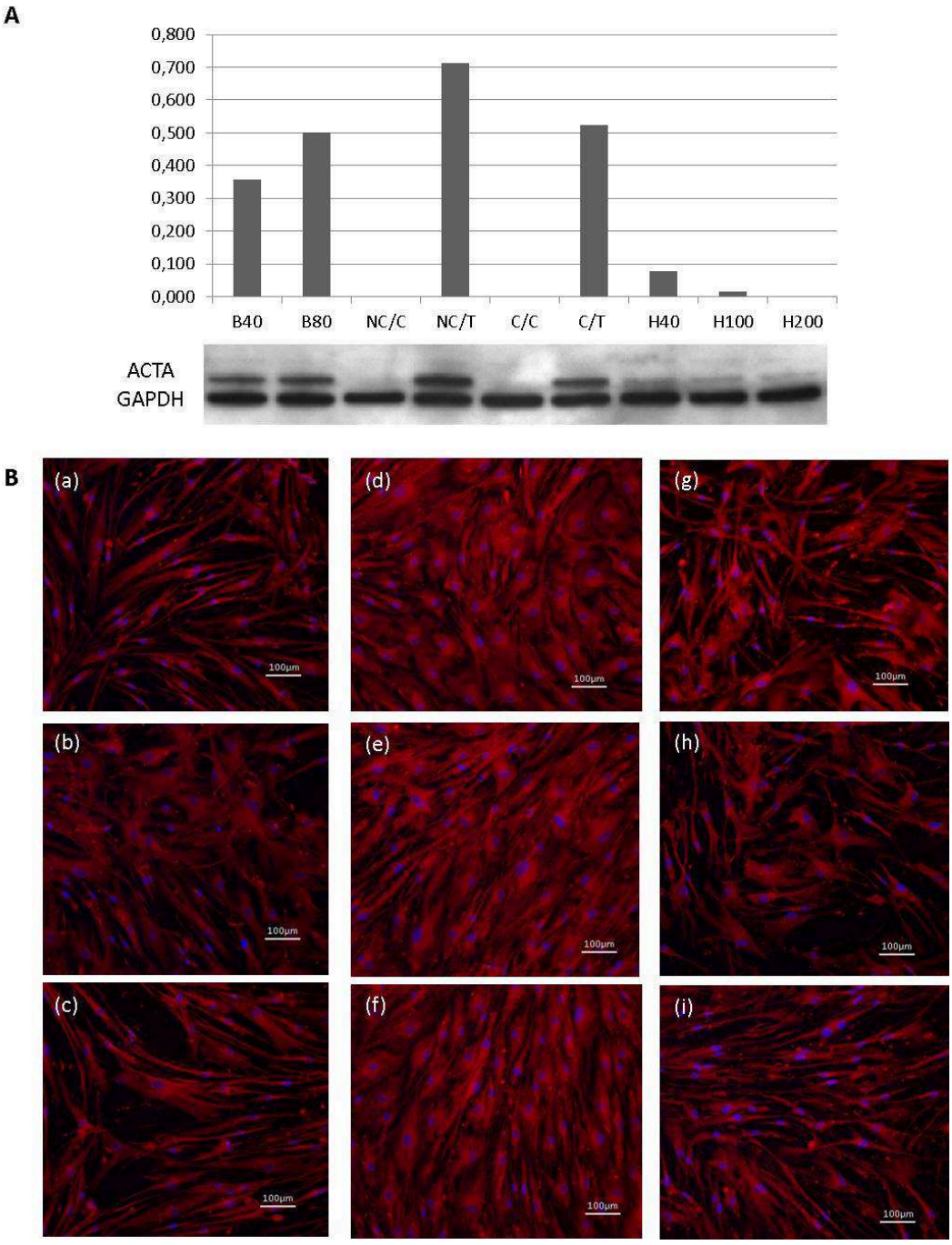

Figure 43: A Westernblot for α-SMA in hVFF; NC/C=non-crowded control; C/C=crowded control; NC/T=non-crowded+TGF-β1; C/T=crowded+TGF-β1; H40=crowded+TGF-β1+HGF40ng/ml; H100=crowded+TGF-β1+HGF100ng/ml; H200=crowded+TGF-β1+HGF200ng/ml; B40=crowded+TGF-β1+Botox40IU/ml; B80=crowded+TGF-β1+Botox80IU/ml; **B** Immunocytochemistry for α-SMA in standard conditions and after treatment with Botox and HGF; (a) non-crowded control; (b) non-crowded+TGF-β1; (c) crowded control; (d) crowded+TGF-β1; (e) crowded+TGF-β1+Botox40IU/ml; (f) crowded+TGF-β1+Botox80IU/ml; (g) crowded+TGF-β1+HGF40ng/ml; (c) crowded+TGF-β1+HGF100ng/ml; (d) crowded+TGF-β1+HGF200ng/ml;

4 DISCUSSION

Regenerative Medicine and tissue engineering techniques, like scaffolding, growth factor injection or stem cell treatment, are slowly approaching clinical routine in our hospitals. Scaffolding for example, as one of the tissue engineering approaches, is meanwhile widely used in the therapy of extensive burn wounds to ensure reepithelialisation of the skin. Big expectations for innovative treatment options with tissue engineering techniques arose also in the area of laryngology, where effective therapeutic strategies for patients with vocal fold scarring are still lacking [14].

In this work a novel in vitro model for vocal fold scarring has been developed with the attempt to accelerate testing for potential antifibrotic agents and to enable closer investigation and modulation of extracellular matrix production by vocal fold fibroblasts. TGF-β was used to induce fibroblast to myofibroblast differentiation and inert macromolecules were added to cell culture to ensure sufficient procession of extracellular matrix. Particular components of extracellular matrix like collagen and fibronectin were shown to be up regulated in the presented model, whereas combination of TGF-β and macromolecules resulted in a further increase of ECM components that exceeded simple addition of single effects from TGF-β and macromolecules. Especially deposition of mature collagen was enhanced in our model which enabled close quantitative and qualitative evaluation. Furthermore HGF and Botox were tested in the presented model for potential antifibrotic properties, whereas only HGF was able to alter ECM production significantly. As the first in the area of laryngology this model offers the possibility for reliable investigation of ECM components, especially collagen, to advance tissue engineering in the field of vocal fold scarring.

As mentioned above, despite big efforts made during the last decade, none of the tissue engineering techniques made its way into laryngological routine so far. The major reason for the tardy development is the difficult testing situation in humans with the smallness of vibratory parts of the larynx and only very limited possibilities of histologic evaluations. So far there is only one trial by Hirano et al. that tested growth factor therapy, representing another tissue engineering approach, with basic

fibroblast growth factor (bFGF) in ten patients with presbyphonia [122]. In this study bFGF showed to be effective with good vibratory properties and acoustic function of the vocal folds at least one year after treatment. Growth factor trials in humans with postoperative scarring however are still missing and are problematic since most surgery on the vocal folds is being performed due to neoplasms and the risk of malignant transformation after growth factor therapy cannot be excluded up to now. However there is one trial by Chhetri et al. in which autologous oral mucosa fibroblasts were injected into scarred vocal folds of five patients [123]. Twelve months later a re-occurrence of the mucosal wave and improved voice parameters were seen in these patients. Although this study represents a big step towards stem cell treatment trials in humans the crucial difference of missing pluripotency of injected fibroblasts cannot be neglected. Same as in growth factors the pluripotency of injected stem cells could probably induce formation of malignant tumors like teratomas for example. Until these concerns are ruled out, big trials in humans and the introduction into clinical routine remain delicate. As a consequence most trials have been performed either in animal models or in vitro.

In most of the animal model studies rabbits [71, 124], dogs [56, 125], pigs [78, 126], mice [127, 128] or rats [129, 130], have been used. Larger animals like pigs, dogs or rabbits offer some advantages concerning especially the investigation of vibratory properties of injured vocal folds [42, 131, 132]. Due to morphological similarities to the human vocal folds and the large size, the larynx of these animals is suitable for investigation of vibratory function in controlled surroundings with conventional methods. Krausert et al. for example studied the vibratory parameters of vocal folds after injury at different locations in excised canine larynges [131]. They recorded vocal fold vibration with a high-speed camera after mounting the excised larynges onto an artificial lung and found that especially injury to the anterior and medial portion of the vocal fold is associated with abnormal vibration. However studies with big animals are elaborative and costly especially in keeping the animals. This might be one of the reasons why most data in this field exist about rats.

Compared to larger animals the rat model is much more cost-effective due to the smaller size and shorter lifespan of the animals. But this is not the only advantage of the rat model since there exist important structural similarities with the human vocal folds. First, the rat vocal fold is also trilayered and second, the composition of fibrous proteins in the lamina propria of rats is also comparable to humans [75]. Moreover with a certain amount of effort even vibratory investigations are doable in rats, as presented by Welham et al. [133]. They developed a rat excised larynx model similar to the one known from canines and tested for the effects of bFGF with promising results. Thinking of further studies on the molecular level, the availability of genome sequence data for rats could be another advantage [134]. Furthermore the formation of chronic vocal fold scar takes up to two months in rats [75], whereas in dogs and rabbits this process seems to endure even six months [71, 124]. This fact prolongs significantly the calculated time span for planned trials.

Another aspect that has been neglected for long when comparing different studies in animal models on this topic is the type of injury to the vocal fold. A variety of instruments like microscissors, microforceps or needles with different gauge have been used by different investigators, creating wounds with various extent and depth [135, 136, 137, 138]. In some studies the surgical method is even only described roughly which seems problematic since healing and thereby fibrogenesis is highly dependent on the underlying injury. Imaizumi et al. proposed in a recent publication a classification system for rat surgery similar to the classification of phonosurgery in humans [139]. Moreover they could depict nicely that a deeper injury of the vocal fold was associated with more pronounced inflammation and extended healing period. This classification could help to improve standardization in animal models for vocal fold scarring.

In conclusion lack of standardization, high costs and extended trial duration in animal models were among others the reasons for the ignition of in vitro trials to gain deeper insights into the scarring process of the vocal folds.

In 2010 Vyas et al. published data about a first in vitro model where they used fibroblasts from normal human vocal fold tissue and induced myofibroblast

differentiation in cell culture with the help of TGF-β in various concentrations [140]. Western-blot was used in this study for evaluation of α-SMA expression of cells, as an indicator for myofibroblast phenotype. The ability of TGF-β to induce fibroblast to myofibroblast differentiation was confirmed in this study. Moreover they used HGF in various concentrations to reverse the effects of TGF-β on fibroblasts successfully. However any effect on extracellular matrix components was not investigated in this particular study.

To evaluate in vitro for extracellular matrix components, especially for collagen, most groups used methods in which they did not measure mature collagen itself but shifted to precursors like procollagen or even to collagen RNA. In a study by Kosinski et al. immortalized human vocal fold fibroblasts were cultured, myofibroblast differentiation was induced by TGF-β and dexamethasone was tested as a potential antifibrotic compound [141]. Notably the evaluation for the antifibrotic effects of dexamethasone was done by quantitative polymerase chain reaction (qPCR) for RNA of collagen 1 and collagen 3, showing a down-regulation of RNA after dexamethasone treatment of cells.

Comparable results have been reported by Suehiro et al. in a study about the effects of basic fibroblast growth factor on rat vocal fold fibroblasts [142]. They investigated in vitro the impact of different concentrations of bFGF on fibroblasts by PCR for procollagen I and procollagen III, whereas results showed a significant downregulation only for procollagen I.

Another interesting study by Kumai et al. investigated modulation of vocal fold scar fibroblasts by adipose-derived stem cells [143]. In an in vitro trial they co-cultured scar fibroblasts from vocal folds of rats with adipose-derived stem cells in transwell plates for six days. ELISA was used to analyse alterations of collagen and hyaluronic acid from supernatants. Cell proliferation and α-SMA were also evaluated. As results they found significant down regulation of collagen, cell proliferation and α-SMA with up regulation of hyaluronic acid.

A similar method was used to analyse the effect of HGF and bFGF on vocal fold fibroblasts from rats of different age in another study [144]. Young and aged rats were used as source for vocal fold fibroblasts, which were then treated in vitro with HGF and/or bFGF. Supernatant of cells was harvested and ELISA was performed to evaluate hyaluronic acid and collagen I content, showing increased production of hyaluronic acid and decreased production of collagen I after treatment with growth factors.

In conclusion, most in vitro trials on potential antifibrotic compounds for vocal fold scarring have evaluated collagen on the RNA level or have been measuring procollagen with ELISA in supernatants of cells. A proper investigation of mature collagen from a representative extracellular matrix has not been performed yet. However, good results have been published with the used methods but only due to the fact that tested antifibrotic agents primarily influence secretion and/or biosynthesis of collagen. Evaluation of agents that interfere with later steps of collagen formation like any inhibitor of C-proteinase would gain misleading results with the proposed methods. In contrast, our in vitro model assures sufficient in vitro production of mature extracellular matrix to assess compounds that interfere with any step in the formation of collagen. A similar model was proposed by Chen et al. using lung fibroblasts [145]. They combined their so called scar-in-a-jar model with quantitative optical analysis of various ECM components and successfully tested substances that inhibit different targets of the collagen biosynthesis pathway. Two other in vitro models with improved collagen deposition have been published by Fish et al. [146] and Clark et al. [147]. In their fibroplasia models hyperconfluent human dermal fibroblasts were used and treated with TGF-β1 to induce fibrotic conditions. Increased collagen deposition in vitro has been proved and inhibitors of C-proteinase have been tested successfully. However the big disadvantage is that sufficient collagen deposition depends on a prolonged culture time with up to one month in their models. In comparison, culture period was five days in our model, resulting in the production of a mature extracellular matrix that has been experimentally altered by the addition of HGF or Botox.

HGF plays a considerable role during embryogenesis, angiogenesis and wound healing and is reported to have significant effects in treating fibrotic conditions in various organs like liver, kidney and lungs [148]. As mentioned above in the specific field of VF scarring various trials in various settings have been dealing with this growth factor and its potential as therapeutic agent. On the mRNA level HGF has been show to increase hyaluronic acid synthase (HAS)-1 and HAS-2, which leads to increased levels of hyaluronic acid and further on to favourable properties of scar tissue [149]. In an animal model HGF has been shown to improve viscoelastic properties and to decrease collagen levels of vocal fold scars after local administration [98]. In another study HGF was locally injected in vocal fold scars of dogs one month after unilateral VF stripping [150]. Six months after initial injury larynges were harvested and effects of HGF were analysed, showing especially decreased levels of elastin. Furthermore collagen density was at normal levels and no tissue contraction was observed. From a functional point of view significant improvements in vibrational patterns were described compared to the untreated sham group. Another goal of the mentioned study was to evaluate different ways of administering HGF. One group was treated with HGF dissolved in conventional PBS and in the other group HGF was released continuously by a gel, whereas favourable results were seen for the gel, as expected [150].

When thinking about potential future clinical application of HGF the question of systemic absorption after topical administration is raised. A study in scarred rat VF attempted to clarify this aspect, showing only minimal transmission into blood and levels that were below detection rate seven days after topical administration of HGF [148]. Furthermore the optimum dose of HGF has to be determined before a potential introduction into clinical routine. However studies dealing with this topic are rare. Suehiro et al. investigated the effects of HGF application in a rat model of aged animals and found favourable effects of a dose of 10 ng/µl in terms of histology, upregulated HAS-2 expression and decreased expression of procollagen-I [151]. Compared to our study, the aforementioned one by Suehiro et al. as well as other in vivo trials used doses that exceeded our used dose by a factor of about 100. On the one hand, the high range of administered doses can be explained at least partially by

the inherent differences of in vitro and in vivo experiments. On the other hand, one must state however that aged VF differ significantly from scarred VF.

In contrast to various other studies performed in vivo and in vitro our investigations could confirm that HGF reduces the amount of collagen significantly. Noteworthy our results were obtained by direct measurement of mature collagen in SDS-PAGE, which is not the case in the majority of competing studies, as mentioned above. However, higher concentrations of HGF (100 ng/ml and 200 ng/ml) did not lead to reduction of total amount of collagen as seen in SDS-Page and immunocytochemistry. This ceiling effect could be explained by the cell growth stimulating effects of HGF, described already in human pancreatic cancer cell lines [152]. To further evaluate this theory we performed cell count analysis which also showed significant increased cell numbers after high dose HGF treatment.

Concerning α-SMA, a dose-dependent effect of HGF could be observed with increasing suppression of α-SMA expression by increasing HGF doses. This puts our findings in line with previous studies in other contexts [28/29]. Noteworthy, results in these circumstances did not differ substantially between TGF-β plus crowding and TGF-β alone, showing that effects were mediated mainly by intracellular signalling and induced by TGF-β.

In another approach antifibrotic effects of Botox type A were evaluated. Clinical observations showed that botulinum toxin type A (BTXA) can improve the eventual appearance of hypertrophic scar and inhibit the growth of hypertrophic scar [153, 154]. Only few in vitro trials about this topic exist, showing inconclusive results. Haubner et al. investigated in vitro cell proliferation and expression of various cytokines and growth factors (Interleukin-6, vascular endothelial growth factor and transforming growth factor-β) after botulinum toxin treatment but did not find favourable results in terms of antifibrotic properties [155]. Likewise, different concentrations of Botox type A did not lead to significant changes in collagen concentrations in our experiments. It is thus tempting to propose that beneficial effects of BTXA on wound healing are predominantly mediated via the neuromuscular

axis (e.g., reduced contraction of muscles in proximity to the wound area) while cell-autonomous effects on fibroblasts might be neglectable.

4.1 Limitations

Limitations of this work arise from the fact that it is an in-vitro model which, even when being close in certain aspects, can never be 100% equivalent to the in-vivo situation. Evaluation of vibratory properties is virtually impossible in this system. Moreover results from testing potential therapeutic agents in our model will have to be confirmed in-vivo, however the number of in-vivo trials could be dramatically reduced with the help of our model. Nevertheless certain aspects could be introduced in the future to the describe in-vitro model in this work to further approximate it to the in-vivo situation. The scarring process of the vocal folds is not limited to the lamina propria but also affects the epithelium which is not represented in our model. From a functional point of view vocal folds are continuously exposed to vibrational stress which has also not been implemented in-vitro so far.

4.2 Future prospect

Adding epithelial cells to our model, preferably in the setting of a 3D cell culture system, would not only better depict the in-vivo vocal fold from a morphological point of view but would also provide the opportunity to closely study the interaction of the involved cell types in the scarring process. Moreover vibrational stress should be applied to the system to represent functional requirements.

4.3 Conclusion

The combination of crowding and TGF-β applied to hVFF showed to effectively enhance in-vitro deposition of major components of extracellular matrix. This mature extracellular matrix allowed for rapid evaluation of qualitative and quantitative effects of potential antifibrotic agents like HGF and Botox, whereas HGF showed to be effective in various concentrations. The aspect of a mature and influenceable extracellular matrix represents a major advantage of this model with the potential to

highly refine research in the field of vocal fold scarring. Thereby antifibrotic strategies could evolve much quicker to broaden the armamentarium to sufficiently treat patients suffering from vocal fold scarring in the future.

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