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REVIEW ARTICLE

Bioreactors for Vocal Fold Tissue Engineering

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It is estimated that almost one-third of the United States population will be affected by a vocal fold (VF) disorder during their lifespan. Promising therapies to treat VF injury and scarring are mostly centered on VF tissue engineering strategies such as the injection of engineered biomaterials and cell therapy. VF tissue engineering, however, is a challenging field as the biomechanical properties, structure, and composition of the VF tissue change upon exposure to mechanical stimulation. As a result, the development of long-term VF treatment strategies relies on the characterization of engineered tissues under a controlled mechanical environment. In this review, we highlight the importance of bioreactors as a powerful tool for VF tissue engineering with a focus on the current state of the art of bioreactors designed to mimic phonation *in vitro.* We discuss the influence of the phonatory environment on the development, function, injury, and healing of the VF tissue and its importance for the development of efficient therapeutic strategies. A concise and comprehensive overview of bioreactor designs, principles, operating parameters, and scalability are presented. An in-depth analysis of VF bioreactor data to date reveals that mechanical stimulation significantly influences cell viability and the expression of proinflammatory and profibrotic genes *in vitro*. Although the precision and accuracy of bioreactors contribute to generating reliable results, diverse gene expression profiles across the literature suggest that future efforts should focus on the standardization of bioreactor parameters to enable direct comparisons between studies.

Keywords: vocal fold, bioreactor, lamina propria, gene expression, fibrosis, inflammation

Impact Statement

We present a comprehensive review of bioreactors for vocal fold (VF) tissue engineering with a focus on the influence of the phonatory environment on the development, function, injury, and healing of the VFs and the importance of mimicking phonation on engineered VF tissues *in vitro*. Furthermore, we put forward a strong argument for the continued development of bioreactors in this area with an emphasis on the standardization of bioreactor designs, principles, operating parameters, and oscillatory regimes to enable comparisons between studies.

Introduction

The human vocal folds (VFs), also known as vocal cords, are located in the larynx and have a unique structure and multilayered composition that enable the production of wavelike motion during sound production as depicted in Figure 1. During phonation, the VFs can sustain frequencies up to 8000 Hertz (Hz) and can oscillate at amplitudes of up to 1 mm.[1,2] Due to their anatomical location, the VFs are susceptible to various internal and external stimuli that can cause damage leading to irreversible changes in structure and ultimately in vibratory function. Voice disorders represent a significant health care challenge that affects millions of Americans each year. Currently, various treatments for voice disorders, including voice therapy, surgery, or biomaterial injections, have shown promise, but none have fully satisfied the recovery of the VFs.[3] While voice therapy may provide temporary relief, in some cases, surgery physically disrupts the native microstructure of the VFs causing scarring.[3–6] Although biomaterial injections tend to cause less scarring than surgery, hydrogel formulations to date fail to emulate the complex interstitial microstructure of the VFs.[3] As a result, full restoration of the VF microstructure has not yet been achieved at the cellular and

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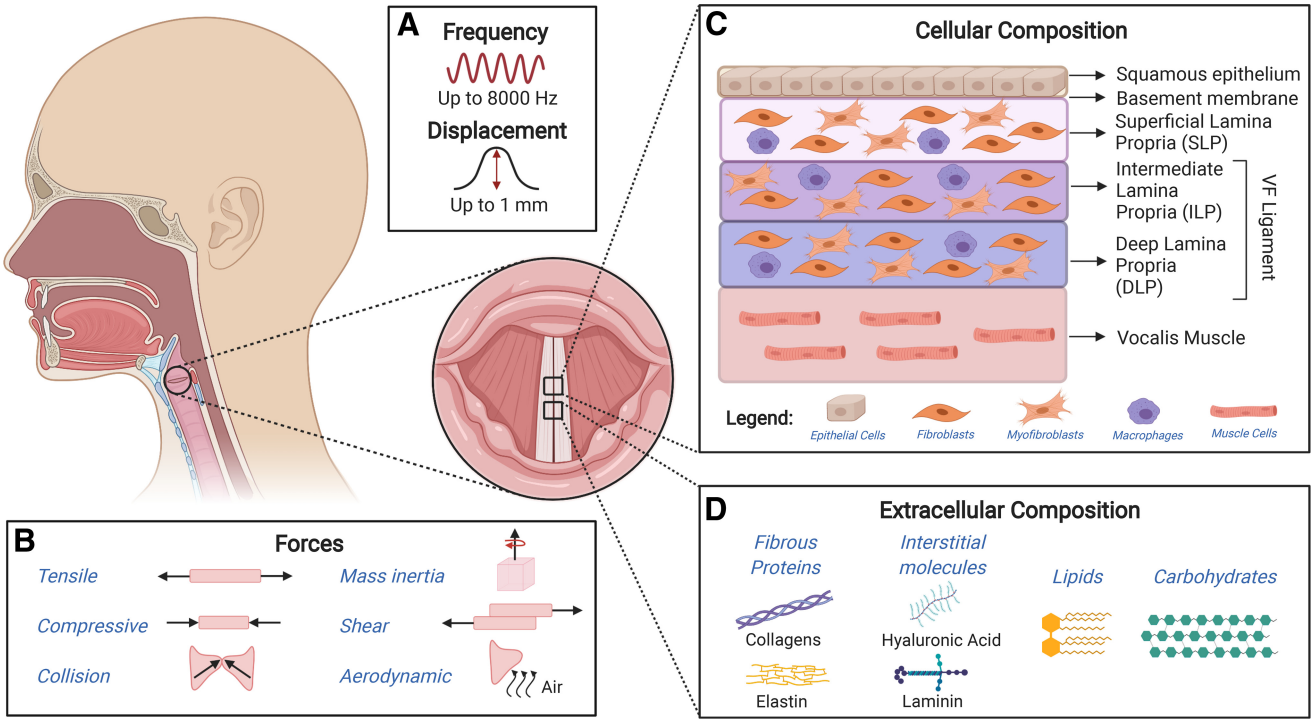

FIG. 1. Overview of the structure, composition, and forces acting on the vocal folds upon phonation. Schematic illustrating **(A)** frequency and displacement, **(B)** forces to which vocal folds undergo during phonation, as well as **(C)** cellular and **(D)** extracellular components of the vocal folds. Color images are available online.

interstitial levels.[3] Promising tissue engineering efforts focus on restoring the interstitial microenvironment of the VFs using synthetic polymers or naturally-derived biomaterials to enable stratified cellular proliferation with the aid of bioactive factors.[7,8]

One challenge in developing treatments for voice disorders is that their long-term outcomes are complicated by the complex biomechanical stimulation, which occurs during phonation.[9] In this context, the use of animal models for preclinical applications is very limited as differences in laryngeal anatomy, phonatory capabilities, tissue structure, and composition largely reduce the scope of studies *in vivo*.[10,11] Although dogs, ferrets, mice, rats, and rabbits have been used in short-term studies, interspecies variability and the inability to simulate the *in vivo* process of human phonation in these animals preclude direct comparisons between studies. Excised larynges have also been used in short-term VF scarring and replacement studies[12–14]; however, simulating human phonation in *ex vivo* models is challenging.[15] Therefore, to fully characterize optimized treatments for voice disorders, it is important to develop and utilize bioreactors that reliably simulate the mechanical and cellular environments of the VFs during injury and healing. In this review, we provide a thorough overview of bioreactor designs that mimic the mechanical phonatory conditions of the VFs *in vitro*.

VF structure and composition

The VFs are composed of three distinct structures: stratified squamous epithelium, lamina propria (LP), and thyroarytenoid (or vocalis) muscle. A thin basement membrane connects the epithelium and the LP.[16,17] The epithelium consists of approximately 5–10 layers of closely packed epithelial cells and its role is to protect the deeper layers of tissue by mitigating the impact of internal and external irritants or mechanical stresses that occur during phonation. The layers of epithelial cells are divided into two regions: basal layer, which is connected to the basement membrane, and suprabasal (or luminal) layer. The basement membrane is primarily composed of collagens; however, other proteins such as fibronectin are also present.[1,18,19]

The LP is a complex trilayered structure subcategorized into the superficial, intermediate, and deep lamina propria. It is heavily involved in sound production and each layer has a distinct structure, molecular composition, and biomechanical properties that determine the vibratory capability of the VFs.[18] The LP primarily consists of extracellular matrix (ECM) components such as fibrous proteins (collagen types I, II, and III, elastin), interstitial molecules (glycosaminoglycans: hyaluronic acid [HA]; proteoglycans: decorin, fibromodulin, and versican; glycoproteins: fibronectin and laminin), lipids, and carbohydrates.[18] A study by Mora-Navarro *et al.* detected ~2000 proteins in a porcine-derived acellular vocal fold lamina proporia extracellular matrix (VFLP-ECM) scaffold.[20] It is important to note that the exact distribution of all ECM components is not currently well understood. In addition, the distribution of various ECM components (e.g., elastins) can vary with age and gender.[21]

Many studies have focused on three important ECM proteins: HA, collagens, and elastins.[1] HA is an example of an important glycosaminoglycan that has gained traction in recent studies as its function goes beyond its osmotic and viscoelastic properties. In addition to its important role in water retention and shock absorption, HA interacts with cell surface receptors enabling cell signaling pathways to regulate its degradation, as well as mediate tissue remodeling

and inflammation.[22] Furthermore, as a biomaterial, its structural versatility permits chemical functionalization into tailored HA derivatives that undergo delayed degradation after injectable delivery.[3] Collagens are fibrous proteins that provide tensile strength, maintain LP organization during vibration, and contribute to tissue remodeling during growth and wound healing.[1,8] Elastins play an important role in VF biomechanics by acting as stretch and recoil proteins during vibration. In the LP, elastins can be found in three different forms: oxytalan, elaunin, and elastic fibers. By intertwining with inelastic collagens fibrils, elastins regulate stretching to prevent damage during injury, while allowing for prolonged cycles of stretching and recoiling without breaking down.[21]

The main cell types found in the LP are fibroblasts, myofibroblasts, and macrophages.[18,20] Fibroblasts are the most abundant cell type and are found in all layers of the LP. VF fibroblasts are responsible for ECM synthesis and play a key role during normal and diseased conditions. Following injury, fibroblasts can activate and differentiate to myofibroblasts to increase ECM synthesis to promote wound healing and tissue regeneration. However, the prolonged presence of myofibroblasts can lead to abnormal overproduction of ECM components, altering the tissue composition and function resulting in scar formation.[20,23] In addition to fibroblasts, macrophages are also heavily involved in orchestrating the complex events involved in VF wound healing and fibrosis.[24] Typically, there is a tendency for proinflammatory (or M1 like) macrophages to appear first at the site of injury, followed by a gradual shift toward prohealing (or M2-like) macrophages.[25] However, the phenotype and distribution of macrophages in the LP layers have not been well characterized.[26]

VF disorders and current therapies

Voice disorders are the most common communication disorder in the United States. Approximately 28 million Americans suffer from voice disorders and it is estimated that 29% of the population will develop a voice disorder during their lifespan.[3] In addition, the cost associated with health care and lost wages approaches $13 billion dollars and is comparable to conditions such as asthma.[27,28] Some examples of voice disorders include contact ulcers, polyps, nodules, cysts, paralysis, cancerous lesions, fibrosis, and so on. Various factors can contribute to voice disorders such as, but not limited to, voice overuse or misuse, gastroesophageal reflux, upper respiratory infections, smoking, radiation, trauma as a result of intubation or surgery, and aging.[3,29]

Many voice disorders, particularly VF fibrosis, represent a challenging therapeutic scenario since they are associated with significant changes in structure, composition, and mechanical properties of the ECM. The VF scar results in overproduction and random deposition of procollagen, collagen, and fibronectin.[20] These changes alter the structure and composition of the VFs, ultimately affecting their vibratory function.

Voice therapy may be used to improve voice quality and reduce vocal fatigue.[3,30] Direct voice therapy is the recommended form of treatment for nontraumatic conditions, such as idiopathic VF paralysis and presbylaryngis, in which the VFs undergo changes in their microstructure resulting in loss of bulk and tonicity.[31,32] Surgical approaches are being used in the form of LP replacement; however, this approach can result in further complications due to high variability in ECM content and biomechanical properties across individuals.[3,30] As an alternative, naturally derived and/or synthetic biomaterial injections [e.g., type I and/or type III collagen, HA, poly(ethylene glycol) (PEG), and porcine urinary bladder matrix] have been used to treat VF injury.[3,17]

Cell therapy is another promising therapeutic strategy focused on the use of mesenchymal stem cells (MSCs) to reduce scar formation. MSCs have shown remarkable regenerative properties in other tissues of the body and are therefore great therapeutic candidates to aid in VF regeneration.[33–37]

The importance of bioreactors for VF tissue engineering

Progress in cell therapy research, biomaterials, and tissue engineering has driven major advances in the field of regenerative medicine. Many of these advancements, however, have been accompanied by low therapeutic efficacy.[38] One way to increase therapeutic efficacy is to simulate the properties of engineered tissues during preclinical trials *in vitro*. However, recapitulating the structure, composition, and metabolism of the VFs *in vitro* is particularly challenging as these factors are altered through mechanical stimulation over time.

Many unanswered questions in the field of VF ontogeny and regeneration would benefit from controlled mechanical stimulation in a VF bioreactor. For instance, there is an ongoing debate in the literature on how different structures of the VF develop throughout life. For example, while most studies propose that the vocal ligament develops after birth with the aid of phonation,[39–41] a study published in 2009[42] suggests that the presence of the vocal ligament at birth may be associated with an individual's genetic profile.[42] Although there is consensus that the LP is present at birth as a uniform nonstratified monolayer,[39,41,43,44] it is not yet known how the newborn is able to cry at near-maximum levels of pitch and loudness without the presence of a fully formed LP.[39] One interesting study aimed to initiate debate in this area, suggesting that the abundance of HA in the newborn VF may facilitate the production of fundamental frequencies ranging from 400 to 600 Hz over extended periods of time without resulting in inflammation or cellular lesions on the site.[39] Current trends in the literature suggest that the development of the microstructure of the LP throughout life is heavily dependent on maturity and the development of phonation and speech.[39,41,45] Studies have shown that the LP starts to differentiate into a bilaminar structure at the age of 2 months and begins to differentiate into a three-layered structure at 11 months, forming three distinct layers by age 7, which undergo interstitial remodeling between ages 11 and 12, and consolidate into a mature, fully formed complex structure through ages 13 and 17.[41,46] This process is not hormonally driven as individuals who are nonphonated from birth or unphonated later in life present hypoplastic VFs without defined VF ligaments and atrophied LP layers, and even the absence of Reinke's space at a mature age.[43] This implies that VF fibroblasts require constant biomechanical stimulation to fully develop the LP and maintain tissue homeostasis.[16]

At the cellular level, it has been suggested that oscillatory stresses can disrupt intracellular adhesions and cellular structures activating mechanotransduction signaling pathways in

fibroblasts.[47,48] In the context of VF engineering, phonatory forces can stimulate proliferation and repair or, depending on the intensity and duration, induce a cell-mediated inflammatory response, which may lead to the formation of a VF lesion.[16]

Several studies have shown that oscillatory regimes play a major role in the fibrotic response of VF tissue constructs *in vitro* by altering the secretion of cytokines, ECM protein concentration, and stiffness.[37,47,49,50] Significant changes in matrix and matrix-related gene expression can also be seen in engineered VF tissues upon exposure to oscillatory motion at different amplitudes and frequencies.[9,34,36,37,47,49–56] ECM remodeling cytokines and HA are also found in greater concentration after phonation.[43,57,58]

Animal studies have shown that experimentally induced phonation significantly upregulates messenger RNA (mRNA) expression of the proinflammatory cytokines interleukin (IL)-1β[57,59] and transforming growth factor (TGF)-β1,[57] as well as the enzymes cyclooxygenase (COX)-2[57] and matrix metalloproteinase (MMP)-1.[59] In agreement with these results, a human subject study showed a significant increase in the secretion of proinflammatory cytokines IL-1β, tumor necrosis factor (TNF)-α, and MMP-8 after 1 h of continuous high-amplitude phonation.[60] Acute edemas and other non-neoplastic lesions have also been linked to episodes of loud phonation and attributed to vasodilation followed by capillary rupture and blood plasma infiltration in a process accompanied by inflammatory cytokine release.[60,61]

Conversely, it has been demonstrated that low impact phonation at the ''resonant voice'' frequency range has an anti-inflammatory and pro-healing effect with significant decrease in levels of the proinflammatory cytokines IL-1β, IL-6, and MMP-8, followed by an increase in the anti-inflammatory cytokine IL-10 after 24 h.[62] Resonant voice frequencies are usually produced through prolonged phonation of /m/, /n/, ''ng'', and /j/ focusing on the production of anterior oral vibrations toward the frontal portion of the larynx.[62] In agreement with other studies, it has been demonstrated that low magnitude cyclic tensile strain suppresses the upregulation of proinflammatory genes in a magnitude-dependent manner, while promoting collagen type I synthesis in the presence of IL-1β.[54]

Given the significant effects of the oscillatory environment on the development and maintenance of VF tissues, *in vitro* systems for VF engineering must be equipped to produce accurate mechanical stimuli, while maintaining cell viability. For this purpose, various bioreactors have been developed. In this review, several bioreactors have been categorized based on the source of vibration into loudspeaker-based (Speaker and Actuator-Based Bioreactors section) (Table 1), actuator-based (Speaker and Actuator-Based Bioreactors section) (Table 2), rheometer-based (Rheometer-Based Bioreactors section) (Table 3), and other (Airflow-Based Bioreactors and Vacuum-Based Bioreactors sections) (Table 4) bioreactors for VF tissue engineering.

Mimicking the Mechanical Environment of the VFs

VF biomechanics

In 1988, Titze[63] demonstrated that the self-sustained oscillation of the VFs was made possible through the propagation of mucosal waves along the LP. More specifically, the interaction between the distinct biomechanical properties of each of the LP layers allows the freedom of motion necessary for self-sustained oscillation.[64] Therefore, the protein composition of each LP layer serves a specific purpose in the propagation of the mucosal wave; for instance, fibrous proteins such as collagen and elastin are responsible for maintaining the shape of the VFs under stress and strain during phonation, whereas interstitial proteins control water content, viscosity, and tissue size, as well as the size and density of the collagen fibers.[64]

Although the biomechanical properties of the VFs are largely determined by the ECM composition of the LP, properties such as viscosity, elasticity, pliability, rigidity, and stiffness can be altered upon changes in VF tension and length during phonation.[18] The anisotropic behavior of the VFs has been verified through stress-strain experiments in which VFs show a nonlinear response during phonation; this nonlinear behavior can be attributed to the mix of collagen and elastin fibers gradually engaging in motion in the anteroposterior and transverse directions of the VFs in a process that promotes changes in VF stiffness and tension.[65]

As a result, therapeutic agents for VF regeneration must incorporate similar properties that sustain this anisotropic, nonlinear behavior, while resisting the dynamic oscillatory environment of the VFs.

Phonation

To date, the most widely accepted theory of phonation is the myoelastic-aerodynamic theory proposed by van den Berg[66] in 1958, in which he postulated that the VFs are actuated by a stream of air delivered by the lungs and trachea.

Physiologically, phonation is the result of highly specialized neuromuscular coordination between the respiratory and laryngeal muscles[67] in a concerted effort to convert the kinetic energy of the subglottic Bernoulli pressure[68] into self-sustained VF oscillation, thereby modulating glottal airflow into acoustic resonance. As a result, the mechano-environment of the VFs is subject to complex fluid-structure-acoustic interactions between the VFs, the glottis, laryngeal muscles, and air being expelled out of the lungs into the upper airways and the vocal tract.[65] More specifically, the VFs are subject to four major mechanical stressors: laryngeal shortening/lengthening, self-sustained oscillation, lateral collision, and air pressure forces in the form of subglottal pressure.

Anatomically, the VFs are located directly above the cricoid cartilage and, within the larynx, are anteriorly attached to the thyroid cartilage and posteriorly attached to the anterolateral surface of the arytenoid cartilages.[65] The laryngeal muscles engaged in phonation interact with the cricothyroid and cricoarytenoid joints, as well as with the cricoid cartilage, changing the geometry, position, and mechanical properties of the VFs.[65] More specifically, the thyroarytenoid and the cricothyroid muscles regulate VF tension by shortening or lengthening the VFs in a process that alters their tension and stiffness.[69] In this context, tension and stiffness are not to be confused as tension refers to the state of physical elongation of the VFs, whereas stiffness is a parameter derived from the VF's biomechanical structure. Even though stiffness is often referred to as an intrinsic property of the VFs, when under mechanical stress, the

Table 1. Speaker-Based Bioreactors

References	Frequency	Displacement	Characterization methods	Forces acting in the system	Cell culture conditions				
					2D or 3D	Cell types	Biomaterials	Exposure time	Cell viability
T.1.A (Zerdoum *et al.*[49])	200 Hz	74 ± 2 μm (mid-membrane); 47 ± 3.6 μm (within gel)	LDV; Digital Image Correlation (Correlated Solutions); FEA (ANSYS software)	Tensile by vibration	3D	Bone marrow-derived hMSCs	HPC gels (HA, PEG, and collagen)	Pilot studies of 5, 15, 30, 60, and 120 min; static 7-day preculture; 1 h on/1 h off for 12 h/day for 3 days	81.0% ± 2.1%
T.1.B (Kirsch *et al.*[56])	Linear chirp; 50-250-50 Hz	Not reported	LDV	Tensile by vibration	2D	Immortalized hVFFs	Pronectin-coated silicone base (BioFlex)	Static 1-day preculture; 8 h off, 16 h of 1 min on/1 min off for total of 2 days	No difference compared to static controls
T.1.C (Tong *et al.*[34])	200 Hz	40 μm	Not reported	Tensile	3D	Bone marrow-derived hMSCs	PCL scaffolds	3-Day static preculture; 1 h on/1 h off for 12 h/day for 3 days, followed by 3 days off	No significant difference compared to static controls
T.1.D (Zerdoum *et al.*[85])	100–300 Hz	40 μm (mid-membrane)	LDV	Tensile	3D	Bone marrow-derived hMSCs	PCL scaffolds and fibronectin	3-Day static culture; 1 h on/1 h off vibration at 200 Hz for 12 h/day for up to 7 days	No loss in viability
T.1.E (Tong *et al.*[36])	100–300 Hz	40 μm (mid-membrane)	LDV	Tensile	3D	Bone marrow-derived hMSCs	Fibronectin-soaked PCL scaffolds inserted into silicone disk	3-Day static preculture; CT culture: 12 h/day for 7 days; OF culture: 1 h on/1 h off for 12 h for 7 days	No significant difference compared to static controls
T.1.F (Farran *et al.*[55])	60–300 Hz	1–30 μm	LDV; tensile measurements of silicone membranes using a Rheometrics mechanical analyzer	Tensile; compressive	2D	Primary human NFFs	Collagen-coated silicone membranes	1-Day static preculture; 1 h followed by 6 h rest	Not reported

CT, continuous; 2D, two dimensional; 3D, three dimensional; FEA, finite element analysis; HA, hyaluronic acid; hMSCs, human mesenchymal stem cells; hVFF, human vocal fold fibroblasts; LDV, laser doppler vibrometry; NFFs, neonatal foreskin fibroblasts; OF, on-off; PCL, poly(ε-caprolactone); PEG, poly(ethylene glycol).

TABLE 2. ACTUATOR-BASED BIOREACTORS

References	*Frequency*	*Displacement*	*Characterization methods*	*Forces acting in the system*	*Cell culture conditions*			*Exposure time*	*Cell viability*
					2D or 3D	*Cell types*	*Biomaterials*		
T.2.A (Bartlett *et al.*[35])	200 Hz	20% Longitudinal tensile strain	Not reported	Tensile by vibration; strain and compression through mechanical actuation	3D	Primary hVFFs (monoculture); human BM-MSC (monoculture); human AT-MSC (monoculture)	Fibronectin-soaked polyether polyurethane scaffolds	2-Day static preculture; 24 h: 12 h at 200 Hz and 20% tensile stress every third minute, 12-h rest	Not reported
T.2.B (Kim *et al.*[52])	205 Hz	19.14 μm at the center; 47.1 μm at 9 mm from center (from Kim *et al.*[53])	Bioreactor characterized in Kim *et al.*[53]	Tensile	2D	hVFFs (monoculture); hMFSCs (monoculture)	BioFlex culture plates	Precultured until confluence (static); 2 s on/ 2 s off for 4 h	hVFFs: no significant difference; hMFSCs: increased viability ($p < 0.05$)
T.2.C (Kim *et al.*[53])	205 Hz	19.14 μm at the center; 47.1 μm at 9 mm from center	LDV	Tensile	2D	hVFFs	Type I collagen-coated BioFlex culture plates	Precultured until confluence (static); 2, 6, or 10 h; 6-h rest	Increased proliferation at 10 h compared to 2 and 6 h of vibration
T.2.D (Bartlett *et al.*[33])	200 Hz	20% Longitudinal tensile strain	Not reported	Tensile by vibration; strain and compression by mechanical actuation	3D	Primary hVFFs (monoculture); human BM-MSC (monoculture); human AT-MSC (monoculture)	Fibronectin-soaked polyether polyurethane scaffolds	2-Day static preculture; 24 h: 12 h at 200 Hz and 20% tensile stress every third minute, 12-h rest	Proliferation or apoptosis did not differ by cell type or mechanical condition
T.2.E (Gaston *et al.*[37])	200 Hz	20% Strain	Not reported	Tensile; impact	3D	Immortalized hVFFs (monoculture); BM-MSC (monoculture)	Fibronectin-soaked Tecoflex strips	Precultured statically until cells attached to the scaffold; 8-h exposure	96% Viability for both hVFFs and BM-MSC

(*continued*)

TABLE 2. (CONTINUED)

References	Frequency	Displacement	Characterization methods	Forces acting in the system	Cell culture conditions			Exposure time	Cell viability
					2D or 3D	Cell types	Biomaterials		
T.2.F (Kutty and Webb[51])	100 Hz	1 mm	Digital stroboscope	Tensile by vibration	3D	NHDF	Methacrylated hyaluronic acid (GMHA) hydrogels cross-linked to Tecoflex films mixed with acrylate-PEG-GRGDS (fibronectin-derived cell adhesion peptide)	Overnight static preculture; 2 s on/2 s off for 4 h/day for 1, 3, 5, and 10 days	<60% Viability
T.2.G (Wolchok *et al.*[47])	100–200 Hz	0.9 mm at 100 Hz for single well; <0.25 mm for six-well plate	Fluorescent microspheres attached to substrates (displacement calculated using an image analysis software)	Tensile by vibration	3D	Human laryngeal fibroblasts	Fibronectin-soaked Tecoflex substrates	2-Day static preculture; 14 min over 6-h period (15-s vibration followed by 30-s rest) followed by 18-h rest; 1-s vibration followed by 2-s rest for 6 h followed by 18-h rest; exposure time varied from 1 to 21 days	Not reported
T.2.H (Titze *et al.*[50])	20–200 Hz	1 mm	Stroboscopic measurements at 1 Hz offset frequency	Tensile	3D	Human laryngeal fibroblasts	Porous substrates (Tecoflex and fibronectin)	3-Day static culture; 6 h of continuous 100 Hz vibration at 20% strain; 100 Hz at 20% strain for 6 h/day for 7 days	>95% Viable; second condition: regions of cell death present

AT-MSC, adipose-derived mesenchymal stromal cells; BM-MSC, bone marrow-derived mesenchymal stromal cells; hMFSCs, human macula flava stellate cells; NHDF, normal human dermal fibroblasts.

Table 3. Rheometer-Based Bioreactors

References	Frequency	Displacement	Characterization methods	Forces acting in the system	Cell culture conditions			Exposure time	Cell viability
					2D or 3D	Cell types	Biomaterials		
T.3.A (Titze *et al.*[83])	100 Hz	1 kPa vibrational stress; 2 kPa oscillatory stress	Real-time feedback by rheometer software (Malvern Instruments)	Oscillatory stresses; shear stresses	2D	hVFF	Fibronectin-treated glass coverslips	Static overnight incubation postseeding; stress regimes: 10 s on/10 s off for 2 h	No quantitative data reported; qualitative observations report low adhesion for substrates seeded with 1000 cells/mm^2, medium adhesion for ~2500 cells/mm^2, and patchy adhesion for 5000 cells/mm^2
T.3.B (Klemuk *et al.*[78])	Up to 150 Hz; tested: 0.1, 1, 10, 35, 43, 67, and 70 Hz	Not reported	Real-time feedback by rheometer software (Malvern Instruments)	Oscillatory stresses; shear stresses	3D	Human tracheal scar fibroblasts (T31)	Fibronectin-soaked Tecoflex substrates	2-Week static culture until cells were elongated across the scaffold pores; 25% duty ratio (45 s on and 135 s off) for 2 h; 75% duty ratio (45 s off and 135 s on) for 2 h	Viability was not affected by vibration conditions

Table 4. Other Bioreactors for Vocal Fold Tissue Engineering

References	*Frequency*	*Displacement*	*Characterization methods*	*Forces acting in the system*	*Cell culture conditions*			*Exposure time*	*Cell viability*
					2D or 3D	*Cell types*	*Biomaterials*		
T.4.A (Latifi *et al.*[9])[a]	~100 Hz	Subglottal pressure of 14.9 cmH_2O	Computational models (SolidWorks and ADINA software); mechanical longevity test; fatigue test; biochemical stability test; rheometry; static subglottal pressure, dynamic subglottal and supraglottal pressure measurements	Tensile; compressive; aerodynamic; impact; inertial; shear	3D	hVFF	VF replicas: silicone rubber (Dragon Skin and Ecoflex) containing hVFF, HA, gelatin, and PEG cross-linker	3-Day static preculture; 2 h/day for 4 days	91.3% ± 2.4%
T.4.B (Branski *et al.*[54])[b]	0.005, 0.05, and 0.5 Hz	3%, 6%, 9%, and 18% cyclic tensile strain	Not reported	Tensile	2D	rVFF	Collagen type I-coated Bioflex II plates	4–5 Days static preculture; 4, 24, 36, and 48 h	>99% Cell viability

[a]Airflow-based bioreactor.
[b]Computer-assisted cyclic vacuum controller-based bioreactor.
rVFF, rabbit vocal fold fibroblasts; VF, vocal fold.

nonlinear structure of the VF promotes changes to the VF's biomechanical structure,[18] thereby changing its stiffness through anisotropic and viscoelastic behaviors.[65]

As the velocity of the subglottal air stream increases as it passes through the glottis, pressure decreases in the vicinity of the LPs, resulting in net energy transfer from the airflow to the VFs.[70] Self-sustained oscillation of the VFs is achieved as they alternate between convergent and divergent conformations in the absence of damping. Damping occurs at the end of a vocal emission as the VFs abduct and cease sound production.[71]

Another important factor in phonation is VF size. VFs vary in size depending on age and gender, typically measuring between 11 and 15 mm lengthwise in adult women and 17 and 21 mm in adult men.[65] As a result, the VFs can be flexible enough to permit oscillatory movements ranging from 50 to 8000 Hz^2 at amplitudes of 0.1–1 mm[50] for varying periods of time. More commonly, however, the VFs vibrate at frequencies ranging from 100 to 1000 Hz.[72]

Bioreactors for VF Tissue Engineering

Bioreactors are designed to act as a controlled environment for cells and tissues to develop *in vitro* by providing favorable conditions, such as optimal temperature, pressure, substrate or scaffold support, regulatory biochemical signals, and physicochemical stimulation.[73] Reliable *in vitro* models or bioreactors for VF tissue engineering must incorporate precise control of four main variables: frequency, amplitude, programmable oscillatory regimes, as well as a suitable environment for prolonged cell viability.[50] In addition to being biocompatible, the materials used in these systems must resist on-off stress regimes and frictional energy dissipation.[50]

In this review, we grouped bioreactors according to working principles described by the original authors. Table 1 lists bioreactors that were described as speaker-based bioreactors, even though, in principle, these are equipped with voice coil actuators to generate oscillatory motion. Table 2 lists actuator-based bioreactors, as they were referred to in the original articles. Table 3 presents a compilation of rheometer-based bioreactors that explore in-plane and out-of-plane shear forces through mechanical and oscillatory stretching, as shown in Figure 2. Finally, Table 4 lists two very distinct principles that rely on air flow perfusion[9] and on vacuum generated cyclic tensile strain,[54] respectively. Figure 2 presents a schematic of the bioreactor principles listed on Tables 1–4 with an emphasis on the types of forces exerted on the scaffolds or substrates in each bioreactor.

Commonly used biomaterials in VF bioreactors

Poly(ε-caprolactone) (PCL) is a Food and Drug Administration (FDA)-approved polymer that has been widely used in the biomedical field due to its biocompatibility, biodegradability, and superior mechanical properties. In the context of VF tissue engineering, PCL has been used as a porous/fibrous scaffold in bioreactors to recapitulate the structure of the VF ligament, as well as an injectable material in the form of microspheres in solution for *in vivo* therapies. One limitation is that PCL is relatively hydrophobic and, as a result, a coating with collagen, fibronectin, fibrin, gelatin, or growth factors is often needed to promote better cell adhesion and proliferation.[3,36,74]

Another commonly used substrate for VF bioreactors is the commercially available BioFlex Culture Plate, a flexible-bottom cell culture plate made of a clear, rubber-like membrane. The plates can be further coated with ECM proteins such as collagen, laminin, elastin, and fibronectin.[75] Tecoflex SG-80A, a commercially available aliphatic polyether-based thermoplastic polyurethane, is also used as a scaffold for VF bioreactors.[76] This nonbiodegradable, medical grade polymer is biocompatible and has been formulated for solution processing. As a polyurethane, Tecoflex has a copolymer backbone comprising of hard and soft segments, imparting elastomeric properties that typically have high tensile and tear strengths with long elongation capabilities.[77] Tecoflex can be solvent casted into a film or porous scaffold with similar properties to the VF ligament and has been used in actuator and rheometer type bioreactors.[37,47,50,51,77,78] Similar to PCL, Tecoflex is not amenable to cell attachment; however, it is slightly hydrophobic allowing for adsorption of proteins like fibronectin.[77]

Fibronectin is one of the most popular and well-characterized biomaterials used to coat scaffolds and substrates in VF bioreactors. Fibronectin's important role in wound healing[79] and ability to bind to other ECM proteins, such as collagen, fibrin, and glycosaminoglycans such as heparin sulfate and HA, makes it the ideal candidate for experimentation *in vitro*.[80] For further information, comprehensive reviews of biomaterials for VF tissue engineering can be found in Li *et al.*[3] and Wrona *et al.*[17]

Speaker- and actuator-based bioreactors

The most popular type of VF bioreactor in the scientific literature is the speaker- or actuator-based bioreactor. Commonly used in cone and flat panel loudspeakers, actuators convert electrical signals into mechanical motion.[81] In a speaker, an electronic circuit digitally controls the current passing through a metallic coil that is connected to a vibrating membrane.[81] The current passing through the metallic coil (also known as a voice coil) generates a magnetic field that interacts with a fixed magnet; the amount of current passing through the coil determines the strength of the magnetic field and its physical position relative to the magnet following the principle of an electric motor and, as a result, the membrane attached to the voice coil oscillates and compresses the air generating sound waves that are proportional to the electrical signal.[82]

This level of digital control allows researchers to accurately program oscillatory routines through hardware-software integration for defined periods of time. In addition, actuator-based bioreactors can be engineered to accommodate modular experimental setups onto which customized cell culture plates can be fitted for multiple replicates. As depicted in Figure 2, most bioreactors described to date use electromagnetic voice coil actuators to mechanically drive oscillation through oscillating air pressure in the form of sound waves (Fig. 2A) or through hard contact (Fig. 2D).[55] Speaker-based bioreactors shown on Table 1 are trending in the literature as these are commercially available, modular, and oftentimes waterproof, and can be run in parallel. Most importantly, they offer programmable routines featuring a

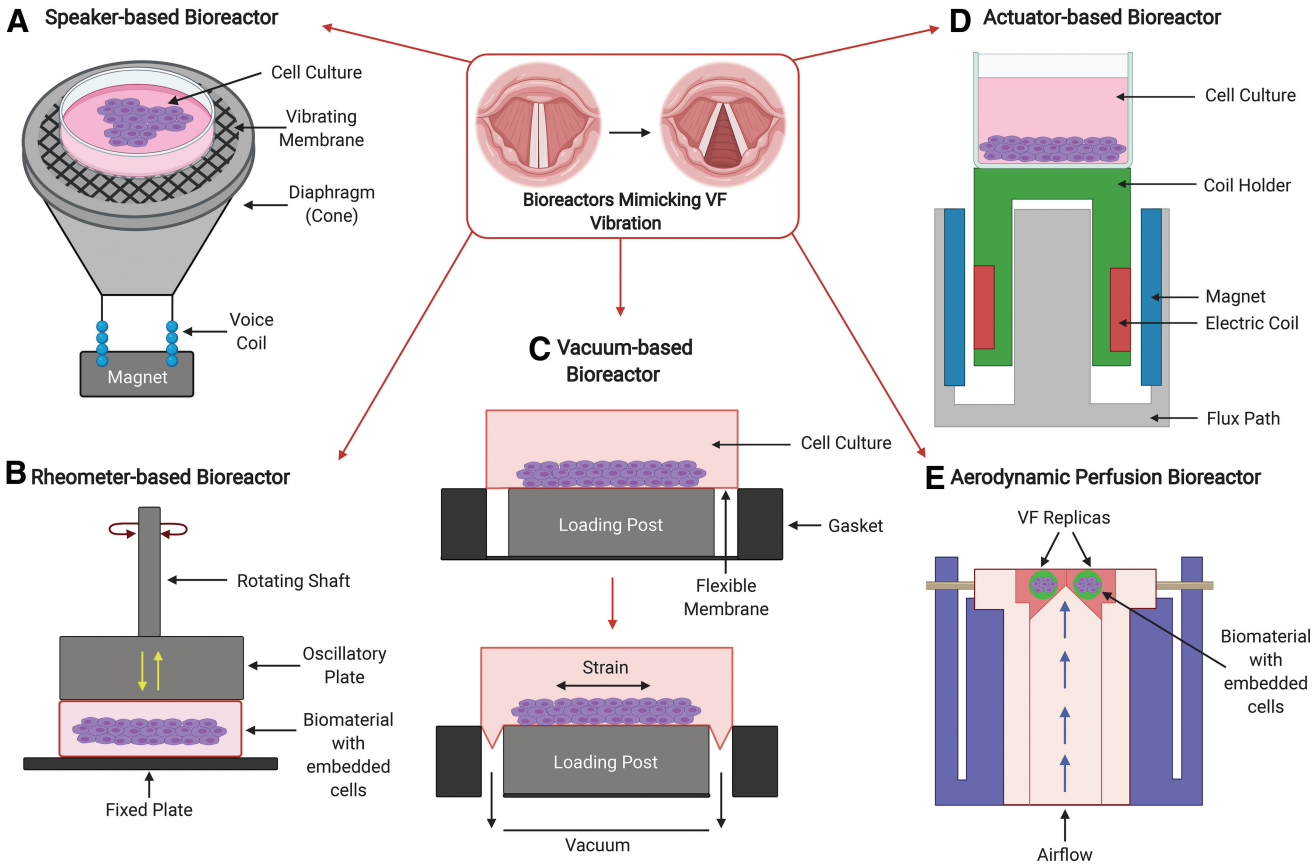

FIG. 2. Schematic of the working principle and forces acting in each type of bioreactor. **(A)** Speaker-based bioreactor principle in which tensile forces are transmitted to the substrate or scaffold by sound waves travelling through the air (Table 1). **(B)** Rheometer-based bioreactor principle in which in-plane stress and strain are applied through rotation around the axis of a piston, which also applies out-of-plane oscillatory forces (Table 3). **(C)** Vacuum-based or cyclical tensile strain bioreactor principle (adapted from Branski *et al.*[54] and www.flexcellint.com/product/transwell-holder) in which a flexible substrate is stretched in all directions with the aid of a vacuum applied beneath the flexible substrate (T.4.B in Table 4). **(D)** Actuator-based bioreactor principle in which the tensile forces are transmitted directly onto the substrate or scaffold through direct contact (Table 2). **(E)** Aerodynamic perfusion bioreactor principle (adapted from Latifi *et al.* 2016) in which the pressurized airflow displaces the substrate or scaffold in the direction of the airflow following the same principle of the myoelastic-aerodynamic theory in which the VFs are actuated by the subglottal airflow (T.4.A in Table 4). VFs, vocal folds. Color images are available online.

wide range of frequencies and amplitudes that can be remotely controlled by Bluetooth or other forms of electronic communication. The main limitation of using speaker-based bioreactors lies in the fact that, depending on the bioreactor design, there may be energy losses in the form of sound waves to the environment. In this regard, actuator-based bioreactors offer an advantage through hard contact with the bioreactor scaffold or substrate. Overall, these two types of bioreactor offer a versatile and easy-to-use option for VF tissue engineering.

Rheometer-based bioreactors

Rheometer-based bioreactors capable of applying lateral sheer and out-of-plane oscillatory forces (Fig. 2B) are also featured in the literature; however, these are less common. Table 3 lists two relatively recent studies that use rheometer-based bioreactors[78,83]; however, these cover a narrower frequency range (<150 Hz) when compared to speaker- and actuator-based bioreactors (<300 Hz). Rheometer platforms tend to be more complex than other types of bioreactors as they rely on heavy instrumentation to guarantee instrumental precision during rotation. This imposes limitations in terms of modularity and size when compared to speaker- and actuator-based bioreactors.

Airflow-based bioreactors

One of the most complex bioreactors incorporating aerodynamic perfusion was proposed by Latifi *et al.* in 2014[84] and more extensively characterized in 2016.[9] This unique study, listed on T.4.A in Table 4, shows that it is possible to incorporate airflow into cell culture to simulate the subglottic pressure and sustain self-oscillation of VF replicas (Fig. 2E). The verisimilitude of this bioreactor with the anatomy of the larynx is highly attractive as a larger number of variables can be incorporated into this design. Such a sophisticated bioreactor design has theoretical advantages over other bioreactor types in terms of types of forces applied to the VF replicas; however, the complexity of its design, fabrication, operation, and maintenance may result in high variability across experimental units.

Vacuum-based bioreactors

Vacuum-based bioreactors are another option for VF engineering as they offer a stretchable and bendable membrane that can be precisely controlled (Fig. 2C). As listed on T.4.B in Table 4, vacuum-based bioreactor studies are rare and tend to operate at lower frequencies (<1 Hz). Although these bioreactors may be available commercially, their operation relies on metered vacuum for precision, which may translate into higher cost and size when compared with other bioreactors.

Parameter considerations

Figure 2 outlines how different forces are exerted on the substrate or scaffold through each method. In effect, the different principles illustrated in Figure 2 can produce very different overall force propagation on the substrate or scaffold as they act through different propagation media and vectors. This has significant implications on the biomechanics of the cells and engineered tissues tested in each bioreactor as the anisotropic behavior of the VF tissue is highly dependent on the magnitude and direction of forces applied to the cells and the ECM. It can be argued that none of the described bioreactors are able to fully recreate all the forces observed *in vivo* as the oscillatory motion of the LP is the result of a multifactorial set of variables that include the nature and direction of the applied forces, propagation media, structure, anatomy, size, thickness, and stretch of the VFs. As a result, bioreactors that incorporate a maximum number of controlled variables are the most promising to study VF disease, healing, and injury *in vitro*.

However, depending on the parameter being investigated, it is possible that certain types of bioreactors may suffice to provide clues on how cells and synthetic biomaterials behave under different regimes of vibration. In this sense, VF bioreactors provide a valuable approximation of the VF mechano-environment, permitting the evaluation of a series of biomaterials, and the viability of cellular cultures and cocultures. At the very least, the structural integrity of novel biomaterials must be tested to survive the vibratory environment of the VFs before clinical translation. The very nature of the VFs calls for dynamic VF tissue engineering methods, regardless of their ability to simulate all forces exerted on the VFs *in vivo*. In addition, the ability to test many experimental units in parallel in a bioreactor is vastly advantageous to evaluate multiple variables at the same time.

In all bioreactors, frequency and displacement were controlled remotely either through an electric switch or computer software. The great majority of bioreactors were characterized using a Laser Doppler vibrometer to evaluate the accuracy of the frequency and displacement being transmitted from the bioreactor core to the scaffold or flexible substrate. Other characterization techniques involved stroboscopic photography, fluorescent particle tracking using a high-speed camera and built-in instrument software. Most articles characterized and described the physical forces acting on the scaffold or flexible substrates as tensile, compressive, aerodynamic, impact, inertial, or shear; mechanical forces exerted on single cells were not reported.

When engineering a bioreactor, one of the most important aspects to consider is the biocompatibility of the materials used. In addition, it is important to establish whether the bioreactor will incorporate fluid flow, gas exchange, and a sterile platform for three-dimensional (3D) or two-dimensional (2D) cell culture. Although 2D and 3D studies seem to be equally prevalent in the literature, 3D cell culture models were the most popular models used in actuator- and speaker-based bioreactors (Tables 1 and 2). The most popular cell types investigated in all types of bioreactors were the human bone marrow-derived mesenchymal stem cells (hBM-MSCs) and a variety of fibroblasts. Among all choices of biocompatible substrates for 3D cell culture, fibronectin-coated Tecoflex was the preferred choice featuring in almost all types of bioreactors, followed by the biodegradable polymers PCL and HPC gels (HA, PEG, and collagen) as flexible scaffolds. The most popular choices of substrate for 2D cell culture were the commercially available Bioflex and Bioflex II flexible culture plates.

2D and 3D cell cultures were exposed to varying oscillatory regimes in all types of bioreactors. Exposure regimes ranged from as little as a total of 5 min a day to as much as 12 h a day. The reported length of the studies varied from 1 to 21 days. Most studies evaluated cell viability of oscillatory versus static cultures at the phenotypic and genotypic levels. More specifically, many studies focused on measuring the gene expression of ECM-related genes to evaluate the effects of oscillatory regimes on ECM-related protein production and remodeling.

Figure 3 shows that most studies overlap with regard to frequency and displacement as it is advantageous to reproduce previously cited parameters for comparison and validation. However, only three studies (T.2.F–H)[47,50,51] portrayed in Figure 3 utilize parameters that are congruent with the common frequency and amplitude range of human phonation (blue region in the graph). Interestingly, although the majority of the studies fall within the most common frequency range of human phonation (100–1000 Hz),[72] most of the listed bioreactors operate at amplitudes or out-of-plane displacements below 60 μm (T.1.A, C, F and T.2.B, C),[34,49,52,53,55] with the exception of three studies that report displacements around 1000 μm (T.2.F–H).[47,50,51] It is important to highlight that most bioreactors described in the literature to date do not reproduce VF tissue stiffness or anysotropy,[85] nor do they incorporate more specialized features capable of simulating the bilateral collision[84,85] of the VFs during phonation, with the exception of attempts by Latifi *et al.* (T.4.A in Table 4).[9]

Bioreactor scalability

Bioreactor capacity is a function of sample size and the total number of experimental units. To maximize experimental reproducibility and reduce cost, experimental bioreactors are usually equipped to process small sample sizes and a limited number of experimental units. Depending on the types of cells and biomaterials involved, it is many times cost prohibitive to run large samples during the early phases of research and development.

In the case of the VF bioreactors reported in the literature, we note that most bioreactors use less than two million cells per experimental unit, with an average number of 10

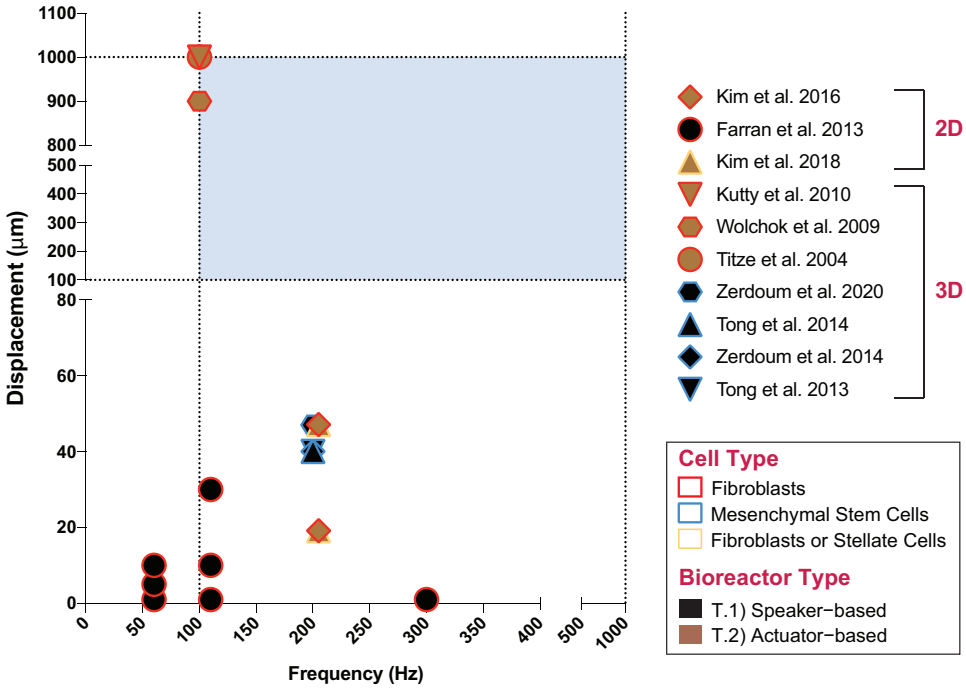

FIG. 3. Cell culture bioreactor parameters. The *blue region* in the graph marks the regions of interest for common human phonation with frequencies ranging between 100 and 1000 Hz at amplitudes or displacements between 100 and 1000 μm. (Graph only includes studies that reported both frequency [Hz] and displacement [μm] values. Studies that reported displacement in different units are not included. Graph only includes values used for cell studies.) Color images are available online.

experimental units per bioreactor as shown in Figure 4. One study by Klemuk *et al.* is an exception to this rule with 16 million cells per experimental unit; however, the authors do not report the total number of units in their bioreactor (T.3.B).[78] Conversely, studies that report the use of very low numbers of cells per experimental unit tend to accommodate a statistically significant number of experimental units in their bioreactors.

The choice of sample size may be important depending on the application of the bioreactor. Larger sample sizes may enable the development of implantable scaffolds or substrates for clinical translation, whereas smaller sample sizes may enable rapid multifactorial screening for the development of new drugs and therapeutics in samples that can be tailored for patient use.

Scalability is an important factor to consider when engineering multiunit VF bioreactors as experimental units may

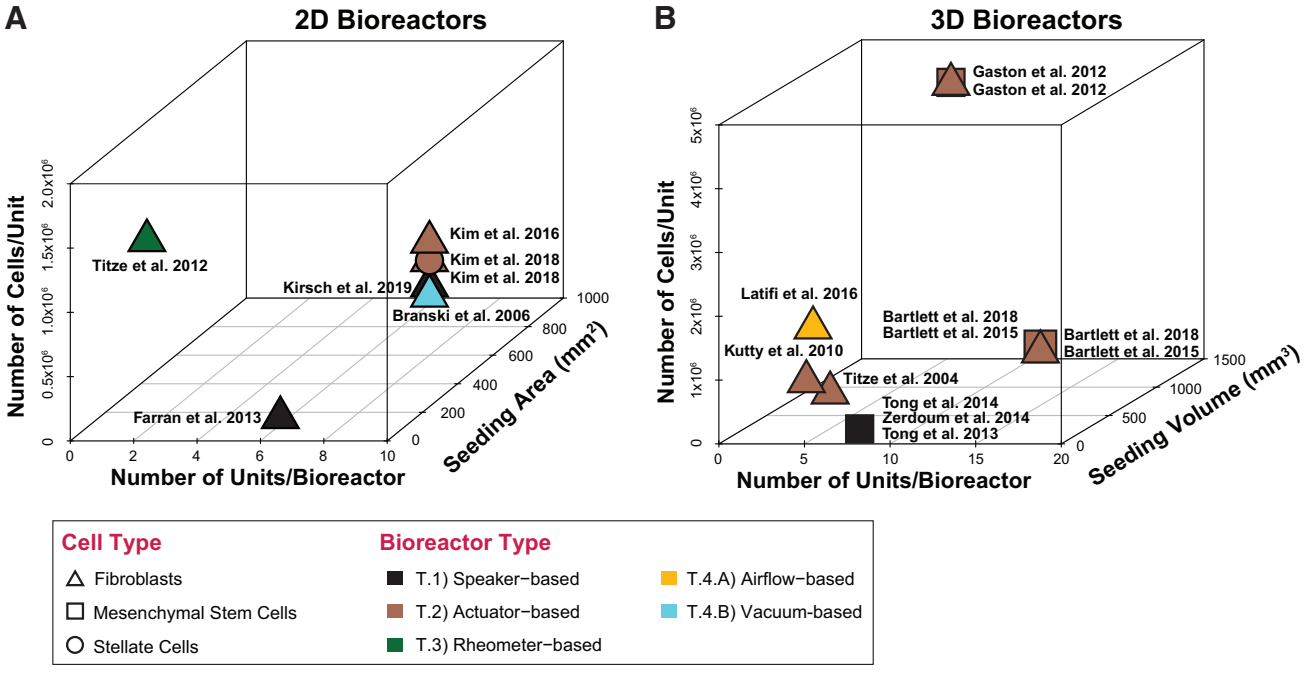

FIG. 4. Bioreactor scalability. **(A)** *XYZ* scatter plot of two-dimensional bioreactors (*X*, number of units/bioreactor; *Y*, seeding area [mm^2]; and *Z*, total number of cells/unit). **(B)** *XYZ* scatter plot of three-dimensional bioreactors (*X*, number of units/bioreactor; *Y*, seeding volume [mm^3]; and *Z*, total number of cells/unit). (Graph only includes studies that reported values for all parameters on the *XYZ* scatter plot.) Color images are available online.

be designed to simultaneously oscillate at precise frequencies and amplitudes. This has important implications for the choice of bioreactor material, design, and engineering, as well as for experimental reproducibility and validity.

Influence on Cellular Behavior

It is a well-known fact that the upregulation of proinflammatory markers correlates with suboptimal healing outcomes at the tissue and organ levels.[86–88] Some of the proinflammatory markers known to interfere with the healing process include cytokines such as IL-1β, TNF-α, prostaglandin E2 (PGE2), and MMPs.[89,90] In addition to inflammation, the upregulation of profibrotic markers such as collagen type I, α smooth muscle actin, and biglycan also hinders optimal healing outcomes.[91]

Figure 5 shows the expression profiles of proinflammatory and profibrotic genes upon exposure to different oscillatory regimes and forces in VF bioreactors. Proinflammatory genes reported in the VF bioreactor literature included *COX2*, *MMP-1*, and *MMP-2*.

COX2 expression was upregulated in studies involving MSCs (T.1.A, E* and **)[36,49] in different culture formats; however, no change in expression was observed in rabbit VF fibroblasts in 2D culture (T.4.B).[54]

MMP-1 expression was predominantly upregulated in MSCs; however, this gene displayed contradictory expression in studies involving fibroblasts showing downregulation (T.1.F)[55] and upregulation (T.2.H)[50] and remaining unchanged (T.1.B; T.2.B*, C, F; T.4.B).[51–54,56] *MMP-2* expression remained unchanged in MSCs in 3D culture (T.1.A).[49]

The expression of the profibrotic gene α smooth muscle actin gene, *aSMA/ACTA2*, was distinct in fibroblasts and MSCs, showing upregulation in human macula flava stellate cells (hMFSCs) (T.2.B**)[52] in 2D culture and downregulation in hBM-MSCs in 3D culture (T.1.A).[49] *aSMA/ACTA2* is one of the key genes involved in fibrosis and its expression remained unchanged in human vocal fold fibroblasts (hVFFs) in 2D culture (T.1.B; T.2.B*),[52,56] and in hBM-MSCs in 3D culture (T.1.E* and **).[36]

Lysyl oxidase (*LOX*) gene expression showed a mixture of upregulation and downregulation in MSCs at different oscillatory regimes, with downregulation seen in oscillatory regimes at 200 Hz and an out-of-plane amplitude of 47 μm that involved 1 h on/1 h off cycles over a 3-day period (T.1.A).[49] Interestingly, *LOX* was upregulated in a 3D MSC culture on a similar oscillatory regime consisting of 1 h on/1 h off cycles, also at 200 Hz and an out-of-plane amplitude of 40 μm, over a period of 7 days, 12 h a day (T.1.E**).[36] Therefore, it appears that the forces applied to the cell culture, as well as the extent and periodicity of exposure to the oscillatory regime play a key role in the *LOX* gene expression. *LOX* upregulation was seen in human laryngeal fibroblasts (hLFs) in 3D culture at intermittent oscillatory regimes at 100 Hz (T.2.G).[47]

The expression of collagen type I alpha-1 chain (*COL1A1*) was shown to be very distinct under different oscillatory regimes. *COL1A1* expression appears to be inextricably linked to oscillatory regimes and cell culture conditions, as this gene showed downregulation (T.1.A, F; T.2.H)[49,50,55] and upregulation (T.1.B, C; T.2.G; T.4.A),[9,34,47,56] and remained unchanged in studies involving fibroblasts (T.2.E, F)[37,51] and MSCs (T.1.E* and **, T.2.E).[36,37]

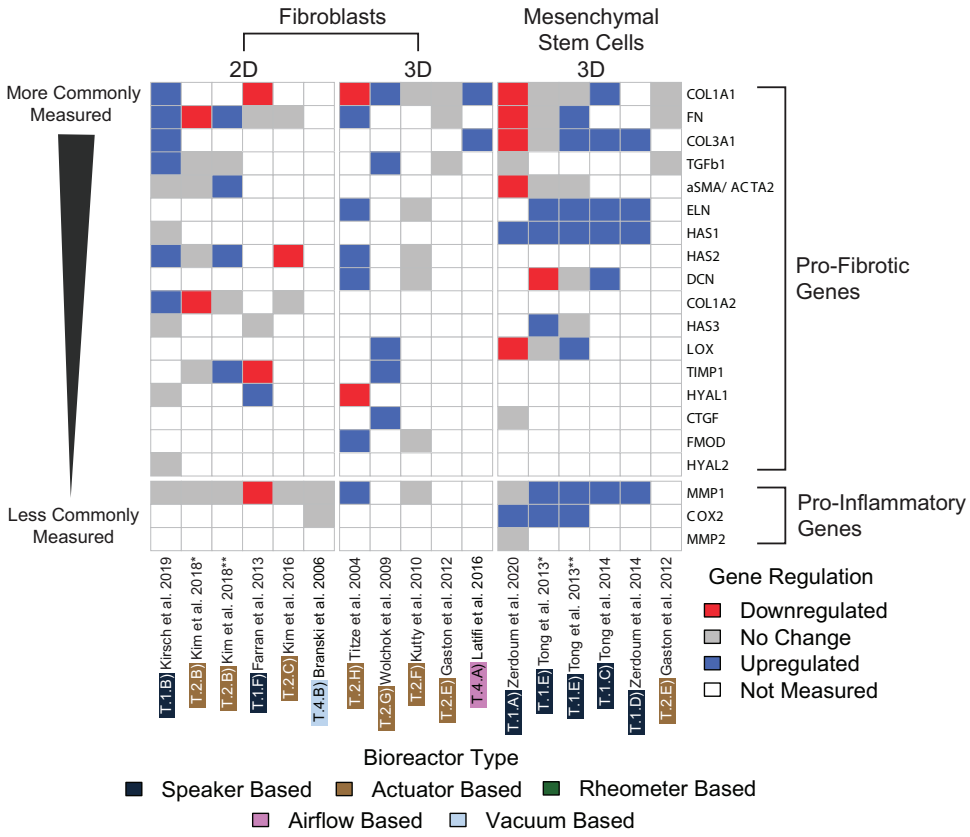

FIG. 5. Gene expression profiles of profibrotic and proinflammatory genes involved in vocal fold tissue engineering. Heat map showing the most commonly measured genes categorized into profibrotic or proinflammatory. Studies have been categorized according to cell type (fibroblasts or mesenchymal stem cells) and cell culture format (2D or 3D). (*Kim *et al.* 2018,[52] hVFF; **Kim *et al.* 2018,[52] hMFSCs; *Tong *et al.* 2013,[36] continuous oscillatory regime; **Tong *et al.* 2013,[36] intermittent oscillatory regime). 2D, two dimensional; hMFSCs, human macula flava stellate cells; hVFF, human vocal fold fibroblast. Color images are available online.

Collagen type I alpha-2 chain (*COL1A2*) expression showed upregulation (T.1.B)[56] in hVFFs in 2D culture. One noteworthy study (T.2.B* and **)[52] that compared 2D cultures under the same oscillatory regime showed that *COL1A2* expression was downregulated in hVFFs, however, remained unchanged in hMFSCs. Collagen type III alpha-1 chain (*COL3A1*) was mostly evaluated in 3D cultures involving MSCs and in only two studies involving 2D (T.1.B)[56] and 3D (T.4.A)[9] fibroblast cultures. *COL3A1* expression was mostly upregulated (T.1.C, D, E**)[34,36,85] in MSCs in 3D culture, except for two studies with very similar oscillatory regimes in which it was downregulated (T.1.A)[49] on an intermittent 1 h on/1 h off regime at 200 Hz with an amplitude of 47 ± 3.6 μm over a period of 3 days and remained unchanged (T.1.E*)[36] on both continuous and intermittent 12-h regimes at 200 Hz with an amplitude of 40 μm over a period of 7 days. Once again, it appears that the periodicity and overall length of exposure seem to play a strong role on gene expression.

The expression of the connective tissue growth factor (*CTGF*) gene that encodes the production of CTGF was upregulated (T.2.G)[47] in hLFs in 3D culture; however, it remained unchanged (T.1.A)[49] in MSCs in 3D culture. The gene responsible for the production of the proteoglycan decorin, *DCN*, was upregulated in hVFFs (T.2.H)[50] and in hBM-MSCs (T.1.C)[34] in 3D culture. In one study (T.1.E* and **),[36] *DCN* expression in hBM-MSCs was downregulated and remained unchanged upon exposure to continuous and intermittent oscillatory regimes, respectively, suggesting that the periodicity of the oscillatory regimes plays a major role on *DCN* expression.

Responsible for regulating the production of the protein elastin, *ELN* expression was upregulated in all 3D cultures involving MSCs (T.1.C–E),[34,36,85] as well as in a 3D culture of hLFs (T.2.H),[50] while remaining unchanged in a 3D culture of hDFs (T.2.F).[51] Another important modulator of fibrosis regulating the production of the protein fibromodulin, the *FMOD* gene was upregulated in 3D cultures involving hLFs (T.2.H),[50] however, showed no change in expression in 3D cultures with hDFs (T.2.F).[51] Fibronectin (*FN*) expression regulates the production of the high molecular weight glycoprotein fibronectin, a key component of the ECM that binds cells to the ECM through integrin receptors. *FN* expression in fibroblasts (2D) showed a mixed response of downregulation (T.2.B*),[52] upregulation (T.1.B; T.2.B**, H),[50,52,56] and unchanged (T.1.F; T.2.C)[53,55] expression. Kim *et al.* 2018 (T.2.B* and **)[52] subjected both hVFFs and hMFSCs in 2D culture to the same experimental conditions and observed downregulation and upregulation of the *FN* gene in these two cell types, respectively. Furthermore, the authors observed that vibrational conditions reduced the stem cell-like properties of hMFSCs, promoting a fibroblast-like phenotype on these cells, concluding that hMFSCs were overall more sensitive to vibration. Tong *et al.* observed that the periodicity of their oscillatory regime resulted in unchanged and upregulated *FN* expression on the same cell types (T.1.E* and **).[36]

The expression of the hyaluronan synthase genes *HAS1*, *HAS2*, and *HAS3* was also evaluated. *HAS1* expression was found to be consistently upregulated in all 3D cultures involving MSCs (T.1.A, C, D, E* and **)[34,36,49,85] and remained unchanged in 2D cultures of hVFFs (T.1.B).[56] *HAS2* expression studies were limited to 2D and 3D fibroblast cultures with very distinct results. Downregulation of *HAS2* was only seen in one instance (T.2.C)[53] involving hVFFs in 2D culture, with upregulation (T.1.B, T.2.B**, T.2.H)[50,52,56] and no change (T.2.B*, T.2.F)[52,53] seen in the remaining studies. *HAS3* expression followed an interesting trend in a study by Tong *et al.* in which hBM-MSCs were exposed to the same frequency and amplitude, however, over different oscillatory regimes, resulting in upregulation upon continuous exposure, versus no change during intermittent exposure (T.1.E* and **).[36] No change was seen in *HAS3* expression in 2D cultures of primary human neonatal foreskin fibroblasts (hNFFs) (T.1.F)[55] and hVFFs (T.1.B).[56]

The *HYAL1* and *HYAL2* genes that regulate the production of the ECM remodeling enzyme hyaluronidase were also monitored in 2D and 3D fibroblast cultures, with the expression of both genes remaining unchanged in hVFFs (T.1.B).[56] *HYAL1* was upregulated in hNFFs (T.1.F)[55] in 2D culture and in hBM-MSCs (T.1.D)[85] in 3D culture, and downregulated in hLFs (T.2.H).[50]

An important modulator of fibrosis, the gene that regulates the production of the profibrotic cytokine, TGF-β1, was evaluated in all modalities of cell culture. *TGF-β1* expression remained unchanged (T.1.A; T.2.E)[37,49] in all MSC cell cultures, however, appeared to be either unchanged (T.2.B* and **, E)[37,52] or upregulated (T.1.B; T.2.G)[47,56] in fibroblast cultures. Gaston *et al.* (T.2.E)[37] compared *TGF-β1* expression in hVFFs and hBM-MSCs under the same conditions and both remained unchanged.

Finally, the expression of the *TIMP1* gene, which regulates the production of the tissue inhibitor of metallopeptidase 1 glycoprotein, was investigated in fibroblasts in 2D and 3D culture. In the same study (T.2.B** and *),[52] *TIMP1* expression was both upregulated (hMFSCs) and unchanged (hVFFs) under the same experimental conditions. *TIMP1* was downregulated in hNFFs (T.1.F)[55] in 2D culture and upregulated in hLFs (T.2.G)[47] in 3D culture.

The data shown in Figure 5 suggest that gene expression does not follow a particular trend for any of the genes under the conditions evaluated. Instead, the data suggest that gene expression is the product of multiple variables, including cell type, cell culture format (2D or 3D), and bioreactor size, as well as frequency, amplitude, periodicity, and overall duration of the oscillatory regime. Even though genes such as *ELN* and *HAS1* showed upregulation in most studies involving MSCs, more investigation is needed. As Tong *et al.* (T.1.E)[36] demonstrated, a particular cell type responds differently to different oscillatory regimes. Kim *et al.* (T.2.B),[52] on the other hand, demonstrated that different cell types respond differently to the same oscillatory regime.

While there are no reported ideal gene expression values for VF regeneration *in vitro*, information on Table 5 may serve as a guide to predict profibrotic or pro-healing outcomes for each gene in the context of VF tissue engineering. Since the VF microenvironment is highly responsive to physicochemical changes, the measurement of gene expression may be a valuable tool to evaluate cell-ECM interactions under static and dynamic conditions and compare tissue response *in vitro* and *in vivo*.

Table 5. Most Studied Proinflammatory and Profibrotic Genes That Play a Significant Role in Vocal Fold Homeostasis

Marker	*Full name*	*Gene*	*Function*	*Expression in healthy tissue*	*Downregulation*	*Upregulation*
COX2	Cytochrome c oxidase subunit 2	*MTCO-2* (mitochondrially encoded cytochrome c oxidase II)	Protein coding gene to produce the COX2 enzyme, which converts AA to prostaglandin endoperoxide H2[93]	Highly restricted[94]	Downregulated in the presence of corticosteroids[94]	Drastically upregulated during inflammation[94]
MMP-1	Matrix metalloproteinase 1	*MMP-1*	Protein coding gene to produce MMP-1 enzyme, which breaks down interstitial collagen type I, II, and III[95]	Low expression is required for postnatal development and tissue remodeling[96]	Inhibited by TIMPs[97] and in the presence of myelodysplastic syndrome[98]	Overexpressed during tissue repair[95] and considered beneficial in the context of VF regeneration.[34] Prolonged overexpression may result in chronic nonhealing wounds.[97] Chronologically upregulated on skin (aging)[99]
MMP-2	Matrix metalloproteinase 2	*MMP-2*	Protein coding gene to produce gelatinase A, a type IV collagenase that breaks down collagen types IV[100] and V,[101,102] elastin, and solubilized monomers of collagens I, II, and III,[103] and inactivates chemoattractant protein-3[104]	Baseline expression is necessary to modulate ECM remodeling, cell growth and migration, inflammation, angiogenesis, and metabolism[104]	Inhibited by TIMP2,[105] doxycycline,[106] statins,[104] and oleic acid (18:1 *cis-9*).[107] Proinflammatory if underexpressed as MMP-2 deficiency leads to cardiac and systemic inflammation[104]	Highly upregulated in connective tissue diseases.[101] Proinflammatory if overexpressed, increasing the deposition of collagen over elastin.[108] Chronologically upregulated on skin (aging)[99]
ACTA2	Actin alpha 2, smooth muscle	*ACTA2*	Gene encoding six distinct globular protein isoforms or actins that assemble into microfilaments. α-smooth muscle actin (α-SMA) is the most studied isoform involved in cellular structural integrity, motility, and intercellular signaling[109]	Baseline expression is necessary to maintain vascular health[110,111]	Inhibited by (TGF)-β-antagonists.[112] Downregulation through antisense mRNA increased cell migration and decreased cell-matrix adhesion[113]	Upregulated in the presence of TGF-β1, increasing fibroblast contractile activity,[114] and stimulating collagen and ECM production[115]
LOX	Lysyl oxidase	*LOX*	Protein coding gene to produce the secretory amine oxidase enzyme LOX, which catalyzes the cross-linking of collagens and elastin[116]	Associated with elastin fiber synthesis in infants and collagen fiber synthesis at all ages[117]	Downregulated in the presence of β-aminopropionitrile (BAPN)[118]	Associated with fibrotic disorders[119] and tumor progression[120]
COL1A1	Collagen type I alpha 1 chain	*COL1A1*	Protein encoding gene for the pro-alpha1 chains of type I collagen, which is a fibril-forming collagen found in most connective tissues and is abundant in bone, cornea, dermis, and tendon[121]	Baseline expression is necessary to protect against soft tissue rupture, including tendons and ligaments,[122] and to maintain bone density and prevent osteoporosis[123]	Downregulation leads to loss of cellular adhesion to ECM components and induces terminal differentiation of keratinocytes.[124] Inhibited during mesenchymal stem cell differentiation into chondrocyte-like cells[125]	Increase in expression in the presence of TGF-β1.[115] Overexpressed in fibrotic tissues[126–129] and tumors[130,131]

(*continued*)

TABLE 5. (CONTINUED)

Marker	*Full name*	*Gene*	*Function*	*Expression in healthy tissue*	*Downregulation*	*Upregulation*
COL1A2	Collagen type I alpha 2 chain	*COL1A2*	Protein coding gene for the pro-alpha2 chain of type I collagen found in most connective tissues and is abundant in bone, cornea, dermis, and tendon[132]	Baseline expression is necessary to maintain ECM integrity and bone density[133]	Downregulated shortly following thermal injury[134,135]	Increased expression has been observed during the proliferative phase of wound healing[134,136] and linked to tumor suppresion[137]
COL3A1	Collagen type III alpha 1 chain	*COL3A1*	Protein coding gene for the pro-alpha1 chains of type III collagen, a fibrillar collagen found in stretchy connective tissues frequently in association with type I collagen[138]	Baseline expression is essential for collagen fibrilogenesis in many organs[139]	Downregulated in the presence of suberoylanilide hydroxamic acid[140] and nintedanib[141]	Upregulated during the proliferative phase of wound healing,[136] profibrotic events,[142–144] and tumor proliferation[145]
CTGF	Cellular communication network factor 2	*CCN2*	Gene that codes the secretion of a matricellular protein that promotes mitosis, also known as connective tissue growth factor, which influences cell adhesion, tissue repair, fibrosis, chondrogenesis, and osteogenesis, and is related to platelet-derived growth factor[146,147]	Baseline expression is required for postnatal fibrogenesis,[148] ECM organization, cell proliferation, migration, and survival[149]	Downregulated during the proliferation and maturation phases of wound healing[150]	Upregulated during the inflammatory phase of wound healing[150] Overexpression leads to failure to stop tissue repair, leading to pathological scarring in fibrosis and cancer[147]
DCN	Decorin	*DCN*	Protein coding gene for the production of decorin, a small leucine-rich proteoglycan that is essential to collagen fibril assembly[151]	Baseline expression levels mediate fibrilogenesis, autophagy, inflammation, angiogenesis, and tumorigenesis[151]	Downregulated in cancerous tissues[152,153]	Upregulation can inhibit fibrosis and scar formation and prevent proliferation and metastasis of tumor cells[154]
ELN	Elastin	*ELN*	Protein coding gene to produce elastin, one of the two components of elastic fibers that confers elasticity to the ECM in tissues and organs[155]	Baseline expression is required during fetal development and for the maintenance of tissue elasticity[156]	Inhibited by basic fibroblast growth factor[157] and interleukin-1β.[158] Downregulation leads to focal stenosis, hypertension, and vascular stiffness[159] and has been linked to aging in mice[160, 161]	Upregulated in the tumor microenvironment[162] and in fibrotic extensible organs such as in chronic obstructive pulmonary disease[163,164]
FMOD	Fibromodulin	*FMOD*	Protein coding gene to produce the small interstitial proteoglycan fibromodulin, which interacts with type I and type II collagen fibrils to inhibit fibrilogenesis[165]	Baseline expression levels are essential for muscle development through normal collagen cross-linkage for the maturation of large diameter collagen fibrils[166]	Downregulation postpones wound closure by delaying dermal cell migration, impeding angiogenesis, and increasing scar formation[167]	Upregulation controls myoblast differentiation[168] and enhances angiogenesis during wound healing.[167] Overexpression has been associated with scarless wound healing[169] and improved tensile strength[166]
FN	Fibronectin 1	*FN1*	Gene that encodes the production of fibronectin, a glycoprotein that is present on the cell surface and in the ECM and is involved in cell adhesion and migration processes[170]	Baseline expression levels are essential for ECM assembly and maintenance[171,172]	Downregulation has been associated with the loss of cellular adhesion sites on the ECM[173] and reduced vascular remodeling *in vivo*[174]	Upregulation is linked to fibrotic outcomes [175,176]

(*continued*)

TABLE 5. (CONTINUED)

Marker	*Full name*	*Gene*	*Function*	*Expression in healthy tissue*	*Downregulation*	*Upregulation*
HAS1	Hyaluronan synthase 1	*HAS1*	Protein coding gene that produces an enzyme that synthesizes hyaluronan or HA found in the ECM. Its functions include joint lubrication, space filling, and enabling cell migration through the ECM[177]	Baseline expression is necessary for tissue development and maintenance[178]	Downregulation is associated with inflammatory and degenerative arthopathies[177]	Upregulation immediately after injury,[179] during wound healing and tissue repair to support blood vessels and fibroblasts.[177] Associated with scarless healing of the VFs[179]
HAS2	Hyaluronan synthase 2	*HAS2*	Protein coding gene that produces an enzyme that synthesizes hyaluronan or HA found in the ECM. Its functions include joint lubrication, space filling, and enabling cell migration through the ECM[180]	Baseline expression is essential for embryonic development and necessary for tissue development and maintenance[178] with low levels detected in normal VF tissue[179]	Downregulation is associated with inflammatory and degenerative arthopathies[177]	Highly upregulated immediately after injury[179]
HAS3	Hyaluronan synthase 3	*HAS3*	Protein coding gene that produces an enzyme that synthesizes the unbranched hyaluronan or HA found in the ECM and responsible for the regulation of hyaluronan synthesis[181]	Baseline expression is necessary for tissue development and maintenance[178]	Downregulation immediately after injury[179]	Upregulated at chronic/and or later stages of wound healing[179]
HYAL1	Hyaluronidase 1	*HYAL1*	Protein coding gene that encodes a lysosomal enzyme, hyaluronidase, which intracellularly degrades HA during cell differentiation, proliferation, and migration[182]	Baseline expression is necessary for the development and maintenance of tissue structure[182]	Downregulation has been linked with tumor progression[183]	Upregulated upon mechanical stimuli[184] and in later stages of wound healing[179]
HYAL2	Hyaluronidase 2	*HYAL2*	Protein coding gene that encodes a weak acid-active form of the enzyme hyaluronidase, which degrades HA during cell differentiation, proliferation, and migration[185]	Baseline expression is necessary for the development and maintenance of tissue structure[182]	Downregulation has been linked with tumor progression[183]	Upregulated upon mechanical stimuli[184] and in later stages of wound healing[179]
TGF-β1	Transforming growth factor beta 1	*TGF-β1*	Protein coding gene that encodes a secreted ligand of the TGF-β superfamily of proteins, which bind to SMAD transcription factors to regulate gene expression involved in cell differentiation, growth, proliferation, and activation of other growth factors[186]	Low baseline expression in cardiac tissue[187]	Inhibited by decorin[188] and hyaluronidase,[189] both natural TGF-β1 antagonists, as well as antisense oligonucleotides[190] and synthetic antagonists.[191,192]	Upregulated in fibroproliferative diseases, acute VF injury (acute inflammation), during the neomatrix deposition following injury, as well as in chronic VF lesions[193]
TIMP1	Tissue inhibitor of metalloproteinases 1	*TIMP1*	Protein coding gene for the production MMP inhibitors involved in cell proliferation and antiapoptotic function[194]	Baseline expression levels are necessary to maintain healthy tissue structure, counter inflammation and apoptosis, and promote cell proliferation[195]	Chronologically downregulated during the aging process contributing to matrix degradation and senescence[99]	Overexpressed in proliferative cancers[196,197] and upregulation has been linked to fibrosis[195,198]

AA, arachidonic acid; COX, cyclooxygenase; CTGF, connective tissue growth factor; ECM, extracellular matrix; LOX, lysyl oxidase; MMP, matrix metalloproteinase; mRNA, messenger RNA; TGF, transforming growth factor.

Conclusions and Future Directions

Bioreactors are essential to replicate the phonatory conditions of the VFs *in vitro*. Efforts to date have largely focused on the development of actuator- and loudspeaker-based bioreactors, with a small number of rheometer-based and aerodynamic-based bioreactors. Studies have mostly focused on the application of tensile forces to cell culture through a variety of oscillatory regimes involving frequencies ranging from 50 to 300 Hz and out-of-plane displacements of 0.1–1.0 mm. Very few bioreactors have explored shear, inertial, aerodynamic, or compressive forces. Most bioreactors utilize between 2 and 16 million cells per experimental condition and incorporate a modular design featuring 3–15 experimental units. Cell types, biomaterials, and substrates vary in the literature, with fibroblasts or MSCs in 3D culture being the most popular choice on porous scaffolds. Cell viability and gene expression on static versus dynamic cultures are commonly measured parameters. Even though the native VFs are composed of multiple cell types such as fibroblasts, myofibroblasts, macrophages, epithelial cells, and muscle cells, all bioreactors presented in this review were capable of incorporating monocultures only. Future studies should focus on incorporating multiple cell types simultaneously to better recapitulate the cellular composition of the native VFs, enabling drug response studies, disease modeling, and the preconditioning of implantable scaffolds or engineered tissues before transplantation.

Although there seem to be trends in the literature, the diversity of oscillatory regimes, cell types, biomaterials, and cell culture formats preclude direct comparisons between similar studies. Future investigations should aim to standardize bioreactor test parameters such as cell type, 2D or 3D culture conditions, frequency, and amplitude, as well as the periodicity and length of the oscillatory regimes to yield comparable data. Experiments should focus on using precision tools to map the forces acting on the system and quantify in-plane and out-of-plane displacement, stress, strain, and shear, as well measure cellular responses through proteomics and gene expression.

Another promising approach to optimize VF engineering in the future is to introduce biomaterials that contain anti-inflammatory cytokines and/or growth factors to stimulate a prohealing phenotype.[20,91] In the case of VF tissue engineering, this strategy can be used synergistically with oscillatory stimulation to upregulate anti-inflammatory and antifibrotic genes in 2D or 3D culture formats.[91,92]

Authors' Contributions

A.M.G.M. contributed with conceptualization, literature search, data analysis, article writing, and editing. A.B. contributed with conceptualization, literature search, data analysis, article writing, and editing. D.S. contributed with conceptualization and data analysis. D.O.F. contributed with conceptualization and article editing.

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