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REVIEW

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The life-cycle and restoration of the human vocal fold

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Abstract

Objective: To better understand the challenges of designing therapies to treat damaged vocal fold lamina propria, it is essential to understand the biophysical and pathophysiological mechanisms involved in vocal fold development, maintenance, injury, and aging. This review critically analyses these points to try and direct future efforts and new strategies toward science-based solutions.

Data Sources & Review Methods: MEDLINE, Ovid Embase, and Wed of Science databases were used to identify relevant literature. A scoping review was performed following the preferred reporting items for systematic reviews and meta-analyses extension for scoping reviews checklist.

Results: The layered arrangement of the vocal fold, develops during early childhood and is maintained during adulthood unless injury occurs. The stellate cells of the macular flava are likely to be important in this process. The capacity for vocal fold regeneration and growth is lost during adulthood and repair results in the deposition of fibrous tissue from resident fibroblasts. With advancing age, viscoelastic tissue declines, possibly due to cell senescence. Strategies aimed at replacing fibrous tissue within the vocal folds must either stimulate resident cells or implant new cells to secrete healthy extracellular protein. Injection of basic fibroblast growth factor is the most widely reported therapy that aims to achieve this.

Conclusions: The pathways involved in vocal fold development, maintenance and aging are incompletely understood. Improved understanding has the potential to identify new treatment targets that could potentially overcome loss of vocal fold vibratory tissue.

KEYWORDS

regenerative medicine, tissue-engineering, vocal fold biology, vocal fold regeneration

Lay summary

This article reviews the scientific processes underlying vocal cord development and changes following injury and aging. The relevance of this to the use of novel therapeutics, aimed at restoring vocal fold vibratory tissue, is then examined.

1 | INTRODUCTION

The vocal fold lamina propria consists of a highly evolved arrangement of extracellular matrix protein (ECMP) that is essential for vocal fold vibration and subsequent clarity of human voice. The arrangement of ECMP can be divided into three layers, termed, superficial, intermediate, and deep lamina propria (Figure 1).[1,2] The superficial layer is the most pliable and consists of small caliber interwoven fibrils of collagen, termed reticular fibers, with interspersed bundles of collagen III and elastin.[2,3] Between this protein network exists proteoglycans, such as decorin and versican, glycosaminoglycans, such as hyaluronic acid and, to a lesser extent, glycoproteins, such as fibronectin that contribute to viscosity.[3–7] The intermediate and deep lamina propria are often termed the vocal ligament with the intermediate layer primarily consisting of elastic fibers and the deeper layer primarily consisting of densely packed fibers of collagen III with elastin content being roughly twice that of human dermis.[1,8,9] Disease or surgical intervention can result in a loss of lamina propria or the deposition of fibrous tissue that alters the finely balanced arrangement of ECMP. This, particularly when occurring within the superficial layer, results in a loss of viscoelasticity and a deleterious effect on voice.

Current solutions to restore lost or damaged lamina propria are suboptimal. As seen in other scarring conditions,[10] treatment runs the risk of triggering recurrent fibrosis and initiating a vicious cycle of scar deposition. A range of novel therapies have been trialed that aim to break this cycle,[11–13] but most remain experimental and in instances where data is available involving human subjects,[14,15] randomized controlled trials are yet to be reported.

To better understand the challenges of designing therapies to address the loss or change in lamina propria structure, it is essential to understand the biophysical and pathophysiological mechanisms involved in the formation, growth and maintenance of the lamina propria. It is also important to examine how the vocal fold ECMP network becomes disrupted and the effects of aging. This review critically analyses these points to try and direct future efforts and new strategies toward science-based solutions.

2 | METHODS

MEDLINE, Ovid Embase, and Wed of Science databases were used to identify relevant literature. A scoping review was performed following the preferred reporting items for systematic reviews and meta-analyses extension for scoping reviews checklist.

3 | RESULTS

3.1 | The vocal fold from birth to adulthood

At birth, the lamina propria of the vocal fold appears immature. Histological analysis indicates there may be some differentiation in the layers of the lamina propria,[16,17] but a clear distinction between the vocal ligament and superficial lamina propria is not apparent.[18,19]

During infancy, reticular fibers, bundles of collagen III and immature elastin can be seen extending from the anterior and posterior part of the vocal fold toward the midline, following existing networks of fibronectin present from birth. In childhood, reticular fibers, collagen III bundles and elastin occupy the length of the vocal fold and as the child matures, the vocal ligament begins to develop.[20] Interestingly, the formation of an adult like lamina propria appears dependent on phonation. An adult that has been un-phonated from birth, does not form a vocal ligament or superficial lamina propria and in turn retains a uniform arrangement of collagen fibers.[21–24] This

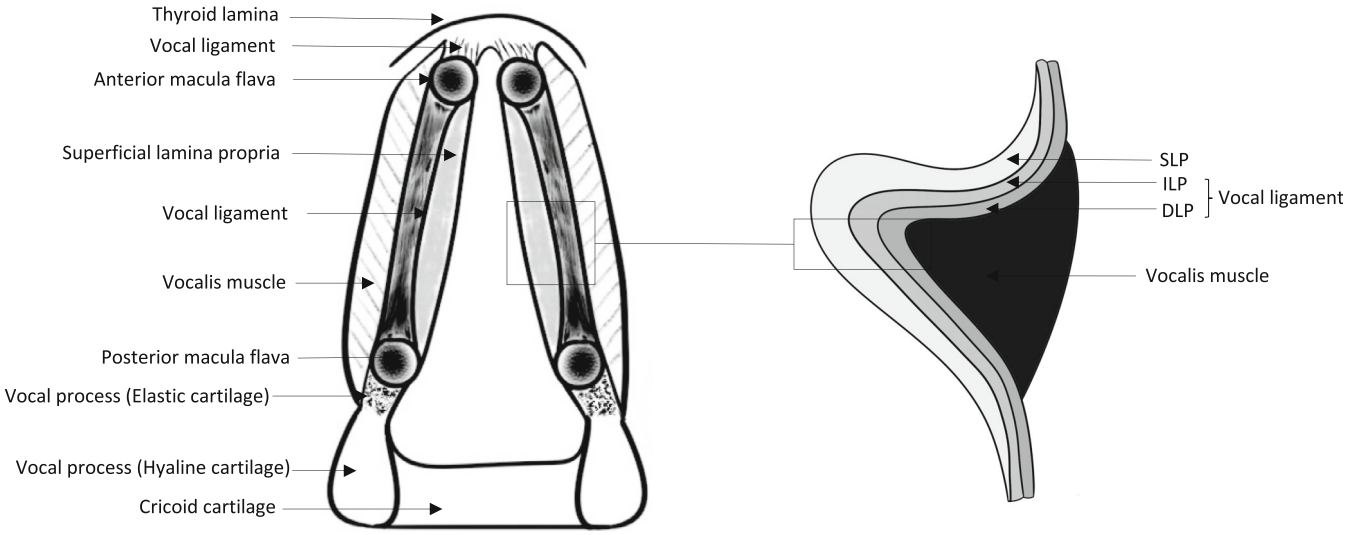

FIGURE 1 An axial and sagittal diagram of the vocal cords. The relevant structures are identified. DLP, deep lamina propria; ILP, intermediate lamina propria; SLP, superficial lamina propria

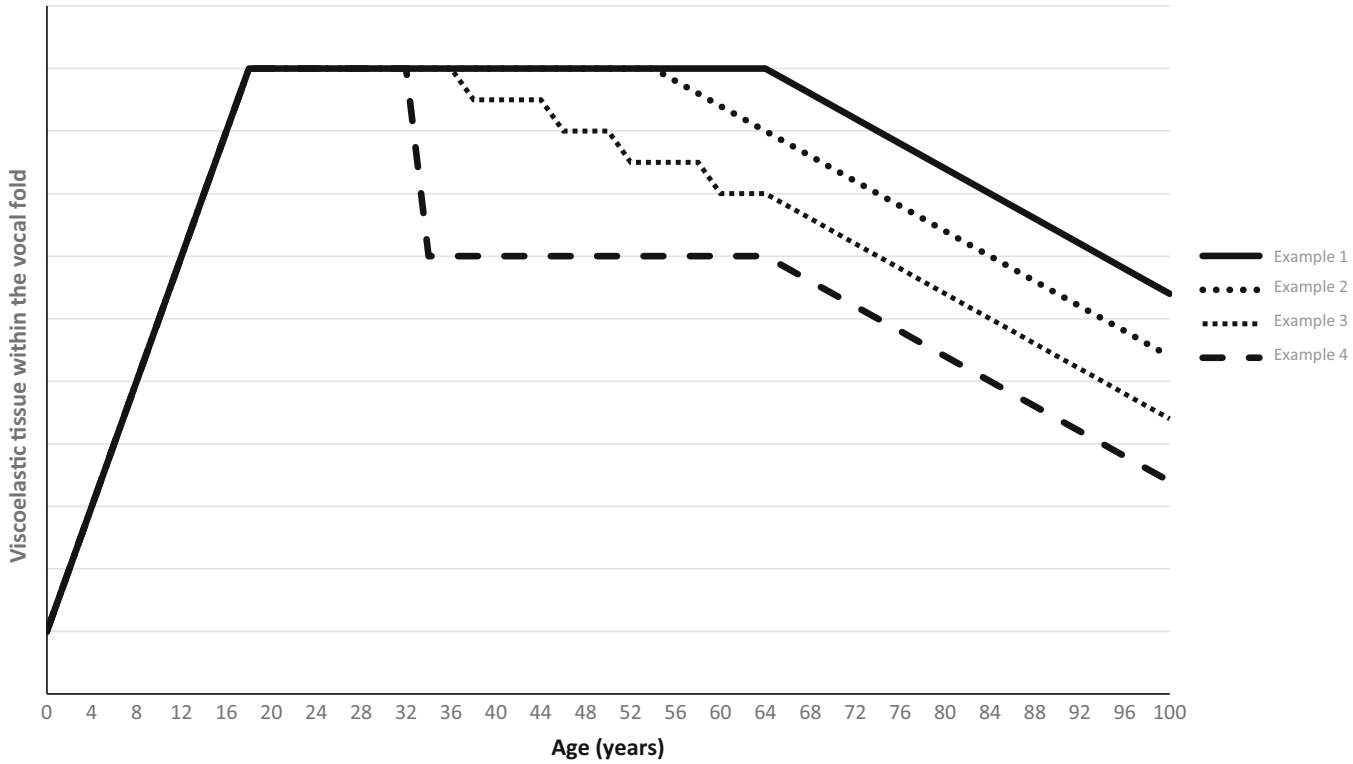

FIGURE 2 The life cycle of the vocal fold is presented. Example one demonstrates a normal life cycle whereby there is expansion of viscoelastic tissue in childhood followed by maintenance in adulthood. As the individual ages, the viscoelastic tissue begins to decline. Example two represents an individual with an early onset decline in viscoelastic tissue due to aging in keeping with presbyphonia. Example three demonstrates sustained and repetitive injury due to voice misuse or factors such as tobacco smoke. Example four represents the life cycle of an individual that suffers damage to the lamina propria resulting in fibrosis or tissue loss. There is a rapid decline in available viscoelastic tissue. Providing there is no further injury, viscoelastic tissue is unlikely to change up to the point where age-related decline occurs. In both example three and four, the effects of aging are likely to have a greater impact on vocal function as there is less viscoelastic tissue to lose at onset.

phenomenon can be explained by mechanotransduction whereby, cells respond to the mechanical cues of phonation to secrete appropriate protein and form the adult vocal fold with a layered ECMP arrangment.[25] This indicates, that at least in childhood, cells exist within the vocal fold that can synthesize and arrange appropriate lamina propria in response to mechanical stimuli (Figure 2).

The location of the cells that synthesize ECMP to form the lamina propria during development are believed by some to be within the macula flava of the vocal fold. The macular flava are $1.5 \times 1.5 \times 1$ mm cell dense regions proximal to the anterior commissure tendon anteriorly and the vocal process posteriorly.[26] The macula flava can be seen on laryngoscopy as yellow colored bulges over the vocal fold at these locations. The majority of cells within the macular flava stain for the hyaluronic acid receptor CD44 and are termed stellate cells.[27,28] Surrounding stellate cells is an abundance of collagen, elastin, glycoprotein, and hyaluronic acid. The density of stellate cells is also highest after birth then declines to reach a plateau in adulthood once the vocal fold has reached maturity.[29] Stellate cells exhibit several stem cell characteristics including the expression of teleromase, colony formation, protein from all three germ cell layers, sensitivity to radiation and a surrounding microenvironment rich in hyaluronan.[30] This has led some authors to identify the macular flava as the stem cell niche of the vocal fold.[31,32] An alternative source of cells responsible for protein synthesis and development of the vocal fold lamina propria are fibroblasts. Fibroblasts are known to be responsive to mechanical cues and secrete and remodel new protein in most stromal tissue.[33] Fibroblasts can also be identified within the lamina propria of an infant's vocal fold. However, unlike stellate cells in the macular flava, cell density within the vocal fold does not change with age as would be expected during periods of growth and development.[28] Unlike stellate cells, fibroblasts within the lamina propria of a new-born do not exhibit characteristics that are conducive to extensive protein synthesis when examined using electron microscopy.[34]

3.2 | Growth of the lamina propria

During puberty, the vocal cords increase in length with growth of the laryngeal cartilage and musculature, and the larynx sits in a lower position relative to the spine.[35] These changes increase the intensity of the voice and lower the fundamental frequency by on average, one octave in males and 3 to 4 semitones in females.[36] The growth of the

vocal cord indicates organized ECMP synthesis during this period, possibly in response to a hormonal stimulus. However, the exact changes to the lamina propria microstructure and the cellular mechanisms responsible for growth are not well understood.

Immunohistochemistry and electron microscopy shows a further maturation of the vocal ligament during puberty with a more clearly defined deep collagenous layer and elastin predominant intermediate layer.[37,38] Optical coherence tomography also indicates the depth of the superficial layer expands during this time while the intermediate and deep layers remain static.[39] Whether hormones involved in puberty directly or indirectly stimulate protein synthesis and organization within the lamina propria is debatable. While sex hormone receptors have been identified in the lamina propria of adult vocal folds,[40,41] other studies have failed to replicate these findings and the arrangement of receptors on vocal fold cells in adolescence is poorly characterized.[42,43] Instead, sex hormones may have an indirect effect by stimulating mesenchymal cells within the vocal folds to secrete growth factors that lead to an upregulation of protein synthesis and matrix remodeling within vocal fold fibroblasts or stellate cells.[43]

Outcomes following voice muscularization with testosterone or voice feminization with testosterone blockers are variable.[44] A lowering of pitch in those receiving testosterone treatment is best correlated with an increase in the length of the vocal tract and thickening of the laryngeal muscles.[45] Currently, there is no evidence supporting a change in protein synthesis or remodeling within the lamina propria following these treatments and suggests, the effect of sex hormones on the lamina propria of the vocal fold is lost or plateaus once the vocal fold reaches maturity.

3.3 | The vocal fold in adulthood

3.3.1 | Homeostasis

The lamina propria of the vocal fold reaches maturity in late adolescence and is fixed in length beyond this age.[35,37] Understanding the mechanism by which the ECMP within the vocal fold can repair damage during homeostasis would provide a potential target for restorative therapies. Observations in skin show that collagen and elastin have half-lives of many decades suggesting a low turnover of protein.[46,47] However, unlike skin, vocal folds undergo repetitive collisions during phonation that potentially, places a higher demand for protein repair and replacement. Hyaluronic acid has a short half-life of 6 h. Given this is the dominant glycosaminoglycans within the superficial layer suggests a mechanism exists by which hyaluronic acid is continually synthesized during homeostasis. Traditionally, fibroblasts within the lamina propria were believed to mediate repair and remodeling of protein under homeostatic conditions. However, more recently, observations that fibroblasts exist in low densities within the lamina propria and exhibit a quiescent phenotype in non-injury states, challenges this belief.[48] Whilst myofibroblasts have been shown to persist within the SLP of healthy vocal folds, and have been reported as indicating myofibroblasts are responsible for ongoing ECMP remodeling, other studies have failed to replicate this finding.[49] Sato et al. have demonstrated that stellate cells within the macular flava continue to show signs of ECMP synthesis during adulthood.[50] Furthermore, markers of ECMP synthesis in the macular flava are lost and the LP atrophies in those that lose the ability to phonate in adulthood.[24] These findings indicate a role for stellate cells in balancing the synthesis of new ECMP and the degradation of old ECMP during homeostasis.

3.3.2 | Injury

Regeneration refers to the replacement of damaged tissue with the same tissue to completely restore organ function. This contrasts with repair which involves the deposition of fibrous tissue to restore partial organ function. The vocal fold lamina propria heals via a reparative pathway following injury in adulthood.[51] Wound repair classically involves three stages: inflammation, new tissue formation, and remodeling.[52] The inflammatory phase is characterized by the formation of a fibrin plug followed by infiltration of immune cells with a predominance of neutrophils and macrophages to scavenge dead tissue and contaminants. New tissue formation starts from Days 2 to 10 and involves fibroblast proliferation, angiogenesis, and the deposition of new matrix protein. Tracking cell division within the vocal fold following injury indicates fibroblasts proliferate from the resident cells within the lamina propria as opposed to circulating cells or cells within the macula flava.[53] These cells go on to secrete collagen and fibronectin with minimal elastin and hyaluronic acid deposition.[54] Fibroblasts then differentiate into myofibroblast leading to contraction of the newly deposited tissue which increases stiffness relative to native lamina propria resulting in a loss of viscoelasticity.[55] The final remodeling phase begins 2–3 weeks after injury and lasts for 1 year or more. It involves the digestion and organization of newly formed protein and, in skin, can lead to a softening of the scarred area. The extent of remodeling within the lamina propria is uncertain. Clinical observation mostly shows no improvement in vocal fold stiffness following fibrotic change and vocal fold nodules and polyps are known to persist in adults without surgical intervention.[56] This contrasts to observations in childhood where most vocal fold nodules resolve during puberty and fibrotic changes secondary to surgery are not always carried through into adulthood.[57,58]

3.4 | The vocal fold in old age

As an individual moves from adulthood to old age, there is a decline in vocal function termed presbyphonia. The extent of the decline is variable with some individuals never appreciating a change in the quality of the voice while others experience pronounced vocal deficit.[59] Histological and electron microscopic analysis of aged vocal folds show a reduction in reticular fibers, an accumulation of dense collagen bundles, areas of fibrous tissue deposition and fragmentation of collagen fibrils.[60] Elastic fibers also become disorganized and fragmented and

form dense masses in the superficial layer.[38,61,62] This reduces the interstitial space between fibers usually filled with glycosaminoglycans and can lead to a loss of an appreciable layered arrangement within the lamina propria. In certain individuals, there is also a loss of volume within the lamina propria, which gives a bowed appearance to the vocal folds. These changes reduce the viscoelasticity of the lamina propria and, in the case of vocal fold atrophy, can result in glottic insufficiency and a dysphonic voice.

Understanding the pathophysiological mechanism underlying presbyphonia is not only important for devising novel therapeutics to treat it but, is also crucial to aid our understanding of the vocal fold life cycle (Figure 2). It is tempting to view aging as a cumulation of "wear and tear" injuries that summate over the course of a lifetime leading to an aged phenotype. However, this model ignores the fine balance between protein synthesis, degradation, repair, and remodeling that occurs throughout the body during adult life. The past 10 years has seen an exponential growth in our understanding of the molecular mechanisms of aging centered around a new understanding of the effects of cell senescence. Cell senescence is characterized by the irreversible arrest of cell proliferation and the enhanced release of a senescence-associated secretory phenotype (SASP).[63] In skin, lung, liver, and kidney, the SASP derived from stromal fibroblasts includes pro-inflammatory cytokines, matrix metalloproteinases that digest ECM and micro-RNA packed in exosomes that can inhibit migration and induce senescence in neighboring cells.[64,65] New evidence suggests, that in skin, the accumulation of senescent fibroblasts in response to extrinsic factors, such as UV radiation, and intrinsic factors, such a telomerase shortening with advancing age, disrupt the delicate balance between ECM degradation and synthesis.[66] This leads to an uncoupling of collagen fibers that change the tension loading of fibroblasts which in turn, induce further senescence, leading to a cycle that disrupts the mechanical integrity of the ECM resulting in the aged phenotype.

Age-related changes in skin and the stroma of endoderm derived organs, such as the lower respiratory tract, show similar ECMP changes to the aged vocal fold lamina propria with an accumulation of collagen bundles, reduced ground substance, disorganized elastic fibers, and fragmented protein fibers.[67,68] Like the skin, the vocal fold is also subject to intrinsic and extrinsic factors, such as smoking and voice misuse, that could drive the accumulation of senescent cells. Stellate cells within the macula flava of aged vocal folds are slender and irregular in shape, show fewer organelles associated with protein synthesis and an accumulation of glycogen particles which is indicative of a senescent state.[62] However, the detection of specific markers of senescence, such as the serine protein inhibitors and anti-apoptotic proteins, are yet to be reported within the vocal fold and should be the subject of future research.

3.5 | The application of novel therapeutics

The application of novel therapeutics to limit the burden of dysfunctional lamina propria can be divided into preventative and restorative measures. For preventative measures to be effective, they must be given within the first 2 weeks of injury before fibrotic tissue is deposited. This limits this approach to a narrow set of clinical circumstances where dysfunction can be predicted, and the treatment can be deployed expediently. In the case of cancer treatment, many anti-fibrotic therapies are contraindicated as, in the case of growth factors, they have potential for oncogenesis, or in the case of implants, they may mask a tumor recurrence.

Most instances involving a loss of vibrating tissue leading to vocal dysfunction are unpredictable and often present to voice specialists many months after onset. This group of patients require restorative therapies that overcome the loss of vibrating tissue by providing new vibrating tissue or restoring the viscoelasticity of the fibrous lamina propria. Restorative therapies can be divided into approaches using implanted material and those that use injectable bio-active substances, such as growth factors or steroid (Figure 3).

3.5.1 | Implantable therapies

Implantable therapies refer to the use of biological, biomimetic or synthetic scaffolds with or without cells or bioactive factors to achieve a restoration of functional lamina propria.

The bio-integration of any implanted therapy lies along a continuum with remodeling and resorption at one end and encapsulation and contraction at the other.[69] The outcomes following remodeling can also diverge and follow either a reparative pathway, whereby the implanted product is replaced by fibrotic tissue, or a regenerative pathway whereby the implanted product is replaced by tissue that exhibits the same biomechanical properties of native tissue. For an implant to be successful it must either undergo remodeling with a regenerative outcome or, possess favorable biomechanical properties that do not degrade following implantation by resisting both remodeling and encapsulation with contraction. Materials that possess the same biomechanical properties as healthy lamina propria but resist remodeling and encapsulation are yet to be reported and therefore the majority of implants described aim to achieve restoration through regenerative remodeling.[70] For this approach to be successful, the implant must influence the inflammatory/immune response away from the native injury response that involves the deposition of fibrous tissue. There are several reports of success in this using hyaluronic acid hydrogel,[71,72] collagen hydrogel,[73] gelatin hydrogel with basic fibroblast growth factor (bFGF),[74] decellulaized bovine vocal fold,[75] and decellularized porcine small intestine submucosa.[76] However, translation of these approaches into human subjects is yet to occur, and in most cases and the molecular pathway by which the material and/or biofactors switch the native reparative fibrotic response to a restorative regenerative response is poorly described.

An alternative approach to relying on the host response to achieve a regenerative outcome is to implant new cells that regenerate healthy tissue. Examples of success in this approach include limbal and macular stem cell implants used in ophthalmic procedures.[77,78] Within the vocal fold, mesenchymal stem cells are most commonly

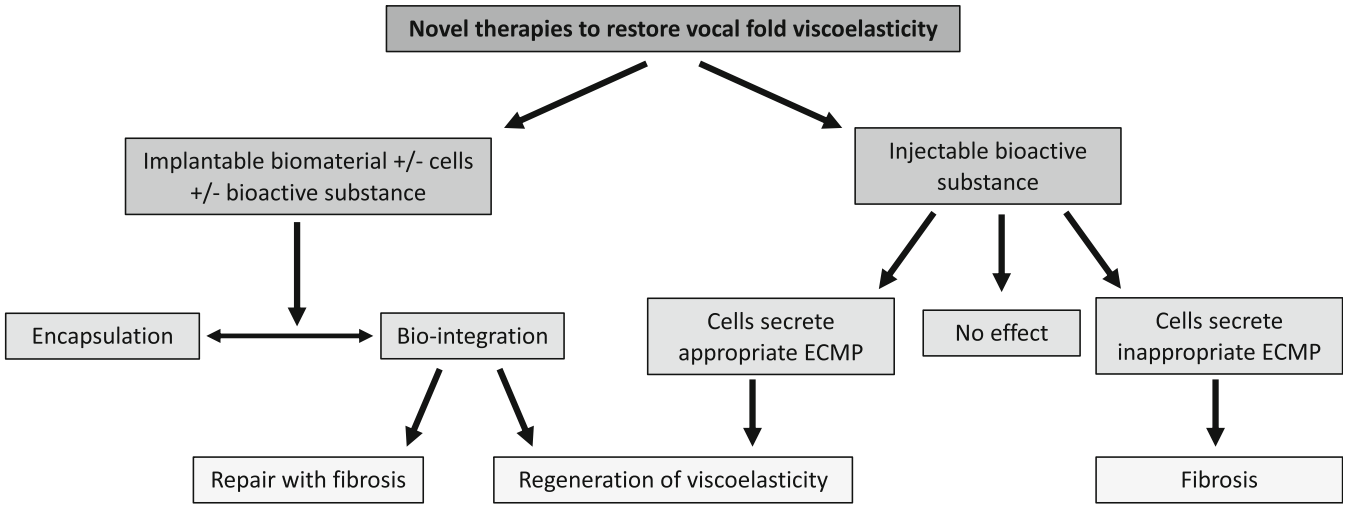

FIGURE 3 A flowchart presenting the pathways for success or failure of novel therapies that aim to restore vocal fold viscoelasticity. Therapies can be divided into implantable and injectable treatments. Any implanted material eventually undergoes encapsulation or remodeling and resorption. These processes can influence the surrounding cellular microenvironment with deposition of viscoelastic extracellular matrix protein that enables restoration of vibration or, deposition of fibrous tissue that does not.

used to restore lamina propria as they retain the capacity for protein secretion, can remodel matrix protein and are believed to reduce the hosts fibrotic response via a paracrine effect involving growth factors and exosome nanoparticles.[12,79,80] Current challenges with this approach include enabling cell survival following implantation with most reports lacking a description of the long-term fate of implanted cells. The use of autologous MSCs such as adipose derived or bone-marrow derived also face challenges with scalability, and therefore adoption as a clinical therapy, due to the expense of autologous cell expansion.

3.5.2 | Injectable bioactive substances

An alternative to implantable therapies are injectable bioactive substances that stimulate cells within the vocal fold to remodel fibrotic lamina propria and/or secrete healthy lamina propria. As we have already seen, under homeostatic conditions, there appears to be a low turnover of ECMP and following injury, repair leads the deposition of fibrous tissue. For injectable bioactive substances to be successful, there must therefore be a phenotypic switch where resident cells are stimulated to remodel fibrous tissue and/or secrete appropriate protein to restore healthy vibrating tissue. This has been demonstrated partially in-vitro whereby, the expression of collagen, hyaluronic acid synthetase and MMPs are upregulated and procollagen I down regulated in vocal fold fibroblasts treated with hepatocyte growth factor (HGF) and/or bFGF.[81,82] In studies comparing young and aged rat vocal fold fibroblasts, HGF only led to an effect in young fibroblasts while bFGF induced matrix remodeling in both young and aged fibroblasts.[83] TGF-β3, a cytokine associated with regenerative healing in fetal skin, has also been used to suppress collagen deposition following vocal fold injury in canines.[84]

Based on these earlier studies, there has been much interest in the use of bFGF as a potential restorative treatment for the vocal fold lamina propria. There are a number of longitudinal studies examining the outcome of bFGF in human subjects with vocal fold atrophy, sulcus vocalis, and vocal fold fibrosis.[15,85–87] Outcome measures from these studies mainly include graded laryngeal stroboscopy, patient reported voice outcomes and voice analysis. To date, all studies have demonstrated a long-lasting improvement in voice outcomes with an excellent safety profile. While these results are certainly encouraging, a placebo double-blinded randomized controlled trial is yet to be performed to confirm efficacy. Unlike surgical procedures where a placebo arm is often unethical, the use of a placebo injection in a patient undergoing a routine examination could be justified given the safety profile of this procedure. The identity of the cells responsible for the effects of bFGF within the vocal fold are still uncertain. Recent advances in genomics, such as single cell RNA sequencing, provides a platform to address this and may highlight alternative pathways, such as a role for the stellate cells within the macular flava. This is clearly important as it would identify new potential therapeutic pathways for drug development.

4 | CONCLUSION

Evidence collated in this article suggests that the lamina propria of the vocal fold forms the layered arrangement crucial for phonation within early childhood in response to vocalization. The vocal cord and lamina propria then undergo a period of hormonally driven growth during early adolescence. In adulthood vocal folds, the capacity for growth or regeneration of lost or fibrotic ECMP appears to be greatly diminished and, the loss of ECMP is managed by the secretion of scar tissue by the surrounding cellular environment. The ability to maintain the

ECMP of the lamina propria appears to decline with advancing age, possibly due to cell senescence. Strategies aimed at replacing scar tissue within the lamina propria must acknowledge these observations and must augment existing cells or implant new cells to bring about a regenerative outcome. Perhaps the most promising approach to achieving this to date is the injection of bFGF into the vocal fold although clinical trials are needed to confirm efficacy.

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CONFLICT OF INTEREST

No financial relationships or conflicts of interest relating to this submission exist.

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