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Tissue engineering of the trachea: what is the hold-up?

Abstract

Tissue-engineering of the trachea is called for based on the clinical consequences of airway pathology. Recent years have witnessed various reports from the tissue-engineering field including a few cases of clinical patients receiving an engineered airway. Multiple fields have accelerated in parallel with the concept of tissue decellularization, isolation of stem cells and possible insights in the biology of different stem cells. Yet, a clinical trial using a tissue-engineered trachea is yet to be initiated. What is the hold-up? Are the hurdles predominantly in the matrix field or cellular regeneration field or both? This systematic review is an attempt to evoke question on what the hold-up might be in the journey of successful regeneration of a functional airway.

Keywords: trachea, tissue engineering, stem cells, matrix

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Abbreviations: HSC, hematopoietic stem cells; MSC, mesenchymal stem cells

Introduction

Embryonic development of the respiratory system is initiated at the level of the primitive foregut, giving rise to the respiratory diverticulum that further develops into formation of the trachea, bronchi and lungs. Essential in this embryonic development is the sequential steps of events at different cell type levels, ultimately leading to stepwise formation and maturation of multiple components of the respiratory system. The process of the development of trachea and lungs starts at 4 weeks. The respiratory epithelium rises from the endodermal part of the respiratory diverticulum, while the connective tissue; cartilage and smooth muscle cells, and rise from the mesodermal part of the diverticulum. Among the major molecular players in respiratory development are FGF10, Wnt, BMP4 and Retinoic Acid that drive Nkx2 and subsequent differentiation and maturation. Specifically, the development of the trachea is driven off the laryngotracheal tube. The laryngotracheal tube consists of endodermal cells that form the inner lining of the trachea controlled by FGF-2 and splanchnic mesoderm forming smooth muscle cells and cartilage by FGF10. The tracheal structure is maintained by a series of 20, C-shaped, cartilage rings that provide rigidity in order to keep the lumen accessible for airflow. These hyaline cartilage rings remain open posteriorly where they are positioned in vicinity of the esophagus and allow esophageal motion. The posterior part of the trachea thus consists of bundles of smooth muscle cells and fibroblastic connective tissue. The flexible area between cartilage rings consists of fibroblastic tissue in continuity with perichondrium to allow flexion of the trachea and elongation upon inspiration. The lamina propria is made up of elastin that provides airway compliance necessary for respiration and vasculature required for oxygen supply and temperature regulation. Tracheal mucosa consists of tall columnar pseudo stratified epithelium with cilia and the mucous producing machinery, the goblet cells. Airway mucosa plays a crucial role in maintenance of airway immunity and flow humidity. The cilia motion and direction are involved in expelling foreign bodies; thereby protecting the delicate lower respiratory system. The upper airway is regulated by mechanical and chemical receptors that contribute to- and perpetuate the protective attribute of the upper airway mucosa by provoking reflexes such as coughing.

In a clinical setting, tracheal pathology remains life threatening and requires rigorous interventional management. Tracheal pathology ranges from traumatic injury to inner and outer tumor formation and inflammatory diseases, leading to tracheal stenosis, thereby causing stridor and respiratory distress. Given the delicate physiologic properties of the airway, it is conceivable that the gradual pressure alteration may lead to delayed and protracted remodeling of the airway system thus affecting patient's performances on a long run. The curative surgical golden standard for tracheal stenosis remains resection of the affected segment. However, the length of the stenosis is a major determinant for surgical decision-making. A stenosis larger than 30% is considered inoperable due to great intra- and post-operative complication risks. Initial steps towards generating a functional airway using collagen sponges in conjunction with a stent or synthetic scaffolds were discouraged due to high rate of graft failure, infections and dissociation of the tissue.[1–4] In 2008, great optimism, enthusiasm and worldwide attention were gained for the first tissue-engineered trachea transplantation by Macchiarini et al.[5] The patient was a 30-year-old woman who suffered from a hypoplastic left main bronchus due to pulmonary tuberculosis. Repetitive stenting appeared unsuccessful and led to recurring pneumonitis. By lack of a better option, tissue-engineered trachea transplantation was considered. The trachea was obtained from a donor, decellularized and seeded with chondrocytes on the outer layer and patient's own epithelial cells on the inner surface. Although the patient has suffered from anatastamotic stenosis and stenting, the tissue-engineered trachea is reported as viable and functional. Subsequently, in 2010 DeLauro et al.[6] Revealed their findings on all transplantation of the trachea in a 55-year-old woman using a cadaveric donor after indirect revascularization in a heterotypic position (forearm fascia) prior to transplantation. The histology of the trachea was built up of a combination of buccal mucosa and patient's own epithelial cells. Finally, in 2011, once again data from the Macchiarini clinic emerged regarding tracheal transplantation in an otherwise inoperable patient using an additional

omental flap to wrap the transplant.[7] Since 2011, no new cases of tracheal transplantation are reported. Evidently, these sporadic cases represent decisions and interventions in the face of clinician and patient desperation and lack of alternative options. Given the age-range of tracheal anomalies and pathologies, more broadly applicable tissue-engineered tracheae are called for. In essence, the concept is unambiguous: exploitation of a matrix that provides stability and stiffness using natural decellularized trachea or synthetic polymers and utilization of peculiar cell types that contribute to formation of adequate histological properties that ultimately cultivate physiologic function. However, the judgment and determination of matrix type and cell type remains an unresolved conundrum.

Materials and methods

Studies were identified using a systematic PubMed search based on title and abstract using synonyms as demonstrated. Our search revealed a total of 42 hits. Manuscripts written in languages other than English were excluded. Nine out of 42 papers were specifically based on regeneration of the lung and were excluded as well. Out of the remaining 31 papers, 5 were review articles that are included and used for references as cited in the text. A total of 26 papers are rigorously reviewed and discussed.

Results

Engineering of organs or organ parts requires accurate imitation and simulation of the natural micro and macro-environment. In the context of the airway, tracheal tissue-engineering can be broken down in multiple particular components that ultimately meet the required criteria for a functional airway: cartilage, smooth muscle cells, extracellular matrix, vasculature and epithelium. The anatomical features of the trachea, its physiologic anterior-posterior difference in rigidity and motion and the blood supply makes the formation of a neo-trachea rather challenging. So far, various fields have investigated various options towards a functional matrix, application of various cell types and vascularization techniques.

Matrices

Contrary to common intuitive assumption, extracellular matrix of a tissue has a prominent role in growth, survival, nutrient supply, maturation of cells, and immune response to pathogens, response to injury and tissue architecture and stiffness. Primitive cells are highly dependent on their inherent tissue geometry in terms of differentiation and development required for tissue homeostasis and function. Shojaie et al.,[8] examined the role of lung extracellular matrix in differentiation of pluripotent cells and demonstrated a robust inductive capacity of the lung matrix on airway epithelial cell differentiation and maturation. In addition, tissue specific cells play an essential part in manufacturing extracellular proteins, formation of matrix and maintenance of tissue structure. This bi-directional cell-matrix cross talk appears to be the quintessence of tissue formation' and the foundation for application of natural decellularized scaffolds that are then seeded with cells. An immaculate scaffold ought to maintain the indigenous three-dimensional structure, yet be free of any cells and MHC components. This intricate balance is rather challenging and requires physical, chemical and enzymatic approaches. The concept of decellularization was introduced in the late 1980's in biopsy material where rat alveolar matrix was prepared to study the role of basement membrane on alveolar epithelial cells.[9] The first successful whole organ decellularization was achieved in 2008 by the group of Doris Taylor demonstrating a 3-dimentional structure of cadaveric hearts, which were repopulated with epithelial and cardiac cells.[10] Momentarily, intricate decellularization protocols have been developed for nearly all organs.[11–23]

Decellularized scaffolds have been utilized extensively in tissue engineering of the trachea. Multiple decellularization protocols have been reported in the past years. A detergent-enzymatic based protocol was utilized in the first clinical transplantation of the trachea where the windpipe is rinsed for 4hours in PBS, stored in distilled water for 48hours at 4C, incubated in 4% sodium deoxycholate for 4hours and an additional DNase treatment for 4hours. This process was repeated for a total of 17 cycles.[5,24] In addition, Remlinger et al. established a detergent-based only protocol of hydrated decellularization and generation of a scaffold. The trachea is incubated in 3% Triton solution for 48hours at 4C. After 48hours per acetic acid and ethanol were added. This process was performed on a mechanical shaker. Due to the triton treatment and application of the mechanical shaker, this protocol is a one-cycle process. Furthermore, another protocol is the treatment of the trachea with a hypotonic Tris-buffer, 1% Triton at 4C for 24hours-48hours on a mechanical shaker. An additional enzymatic treatment with DNase/RNase is applied for 24hours. This mechanical detergent-enzymatic protocol requires solely one cycle. Haykal et al.,[25] conducted a comprehensive comparative study between the protocols and concluded that the 17cycle protocol caused a significant decrease in mucosal and cartilaginous components of the native trachea. In addition, the first and second protocols appear to cause an increase in compliance, thereby reducing the reliability on long-term maintenance of graft integrity, while tracheae treated with the third protocol, appeared more comparable to the native trachea. An additional interesting finding was that none of the mentioned protocols eliminated MHC components entirely in sub mucosal compartments, which indicates that none of the decellularization protocols succeed in generating a non-immunogenic graft Undesirably, for the sake of consistency within this comparative study, the one-cycle protocols were conducted in 17 cycles as well. This, however, does impact upon the characteristics of the graft, given the fact that in experimental settings, these protocols are applied only once.

Further studies on decellularized tracheal integrity revealed that the detergent-enzymatic processing leads to biochemical alteration of cadaveric trachea that may compromise mechanical properties of scaffold due to a reduction in glycosaminoglycans leading to declined tensile strength.[26] On the other hand, processing of rat tracheal scaffolds using detergent-enzymatic treatments appeared to not influence the mechanical strength of the scaffold, despite alteration of extracellular matrix composition *in vitro*.[27] However, subsequent *in vivo* application of the decellularized rat trachea supported epithelilatizatin but failed to host engraftment of stem cell-derived chondrocytes.[28] An additional, extensive *in vitro* and *in vivo* study conducted in rats using rabbit donors revealed that detergent-enzymatic method provides a scaffold similar to native trachea *in vitro* were resistant to collapse after 15 and 30days of transplantation. Additional efforts have been invested in further optimization of decellularization protocol by addition of ingredients such as Genipin.[29,30] Despite an ostensibly phylogeny and accelerated development and advancements in decellularization protocols, graft failure and collapse, breakdown and tissue dissociation are not exceptional.[31] Parallel to the field of natural scaffolds, a plethora of creative inventions were ignited in the field of polymers scaffolds and alternative biocompatible and biodegradable natural sources such as xenogeny extracellular matrix. Application of chondrocytes and mesenchymal stem cells on a porcine cartilage powder scaffold has been reported previously

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with promising cell survival and differentiation outcomes in rabbits. However, future long-term qualitative and quantitative data are yet to be published.[32] In addition, expertise has been gained in hydrogel based matrix application; fibrin/hyaluronan composite gel and various other collagen-based polymers.[33,34] Despite this major gain of momentum in the field of polymers, extensive and protracted future studies are required for accurate assessment of safety and efficacy of these scaffolds. It is worth emphasizing that, based on the literature from the past 10years, interpretation of pre-clinical work on tracheal decellularization and application of polymer based scaffolds require rigorous contemplation of the variability and differences between studies as far as animal models, number of cycles, duration of treatments, the seeded cell type, source of matrix substance etc. A major lack of consistency, unfortunately, remains palpable.

Cell sources

The main sources of cells used in tissue-engineering of the trachea are mesenchymal stem cells (MSC), mesenchymal stem cell-derived chondrocytes and hematopoietic stem cells (HSC). MSCs are multipotent *in vitro* and hold the potential to differentiate into a chondrogenic lineage, osteogenic lineage and adipogenic lineage. Lineage specificity is, strictly, dependent upon interaction and adherence to extracellular matrix driven by adequate cocktail of growth factors. HSCs, driven from the bone marrow, are softer in nature and are less adherent *in vitro*. *In vivo*, however, adherence appears mandatory and is highly reliant on surface stiffness and exposure to growth factors of specific niches. Initial clinical application of tissue-engineered trachea was based on mononuclear cells from bone marrow further induced by growth factors such as EPO and GSCF. The ultimate clinical outcomes are yet to be decoded and re-interpretated. Additional efforts were invested in animal models to study, identify and characterize the mononuclear population and effects of growth factors in tracheal engineering;[35] however the results remain controversial and unfold. An alternative prevailing approach has been the application of mesenchymal stem cell-derived chondrocytes.[27,36,37] Despite the major advantages of clinical availability and processing protocols, results remain unsatisfactory. Since mesenchymal stem cells reside in adipose tissue and provide an effortless source for harvest, multiple studies have attempted to use adipose-derived mesenchymal stem cells.[31,38] Foreseeable, the models have been either discouraging or not interpretable due to low number of animals. A refreshing approach was chosen by Gray et al.,[39] by using amniotic mesenchymal stem cells in 13 fetal lambs with airway defects. The constructs appeared to undergo enhanced remodeling and epithelialization *in vivo* with promising alternatives for repair of inner airway surface. Unfortunately, tracheal connective tissue formation was not included in the study. Due to prominent variability in the choice of cell source, a comprehensive study has been conducted on epigenetic comparison of diverse MSC sources, resemblance and dissimilarities. Four commonly used MSCs sources, bone-marrow, adipose tissue, umbilical cord and skin, were analyzed in detail as far as morphology, Immunophenotypic and differentiation. Reinisch et al performed a genome-wide methylation, transcription and *in vivo* evaluation of MSCs obtained from various sources. Solely BM-MSCs appeared potent of differentiation towards an angiogenic-chondrogenic lineage, mimicking the formation of marrow cavity during embryonic development. BM-MSCs displayed a chondrogenic genetic and epigenetic blueprint. Expression of genes such as RUNX3, RUNX2, MMP13, and ITGA10 were evident indicating early skeletal development. MSCs harvested from adipose tissue, umbilical cord and skin, exhibited clear morphological resemblance to BM-MSCs and yet appeared distinguishable on genetic and epigenetic attributes.[40] In addition, since the discovery by Shinya Yamanaka and emergence of induced pluripotent stem cells, application of iPSCs in tracheal regeneration seems inevitable. However, so far, no major iPSC-based studies have been reported. The conundrum in cell choice for tracheal tissue-engineering may root in lack of comparative studies such as Reinisch et al.[40] After all, the fundament of tissue-engineering is based upon generating neo-tissue by imitating natural development. BM-MSCs appear immaculately capable of bone-marrow cavity formation and engraftment of HSCs in a vascularized micro-environment, perhaps due to natural predisposition during embryonic development. Based on the literature so far, no rigorous search has been conducted in sources that ought to carry morphologic, genetic and epigenetic predisposition for airway development.

Epilogue

In the year 1993, Langer and Vacanti defined tissue-engineering as 'interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain or improve tissue function'. Langer and Vacanti's article in Science then subdivided tissue-engineering fields based on embryonic layers; ectoderm, endoderm and mesoderm as fundamental basis for understanding tissue of origin in order to mimic or imitate tissue properties. A glimpse at embryonic development of the trachea and current literature leaves scientists perplexed. Bone Marrow Mononuclear Cells have not been described in tracheal embryonic development, nor has stimulation using EPO. Bone-marrow, indeed has a mesodermal origin, however committed BM-derived cells are less likely to exhibit potentials for a demanding task in regenerating cartilage. Similar arguments hold true for adipose derived MSCs etc. The delicate study by Reinisch et al. may carry a broader message than the chondrogenic potential of BM-MSCs. MSCs from various tissues carry dissimilar properties, indicating that source matters no matter what tissue is desired to be engineered. In addition, in the field of tissue-engineering of the trachea, search terms such as transcription factors, gene expression and epigenetic characterization seem largely neglected. Ivanosvka et al recently published a comprehensive intriguing review on Cell-Matrix interaction; the effect of matrix on cells and cells on matrix (Ivanosvka 2015), description of matrix proteins, and adhesive properties of cells and most importantly, the cellular needs for particular tissue stiffness that derives cell growth, differentiation and maturation. None of these aspects appear prominent or addressed in engineering a functional airway. Although major advancements have been gained in decellularization protocols, graft failures have been reported repeatedly (ref). While more and more efforts are invested in optimization of these protocols, no rigorous studies are conducted in elucidating the ability of seeded cells in maintenance of the matrix and production of extracellular proteins such as in the natural environment. One could argue that graft failure may not solely be due to failed decellularization protocol but represent an equivalent of a patient lacking stem cells who fails to maintain tissue architecture and display a pre-mature aged phenotype. It is conceivable though that in 1993 Vacanti and Langer were not exposed to such high pressure of 'translation research' by patients, clinicians and funding organization. Other than elite laboratories, contemporary science may suffer from the allure of 'translational research', thereby taking advantage of convenient and available sources rather than sources based on fundamental biological properties and embryonic origin. Perhaps the hold-up is enthusiasm, urgency and impatience thereby making things as simple as possible but then just a little simpler.

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Conflict of interest

The author declares no conflict of interest.

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