Tissue Engineering Auricular Cartilage: A Review of Auricular Cartilage Characteristics and Current Techniques for Auricular Reconstruction

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Abstract: Microtia and anotia are congenital auricular anomalies that negatively impact the psychosocial development of those affected. Because auricular cartilage is a type of elastic cartilage that lacks regenerative capacity, any notable defect in its structure requires a surgical approach to reconstructing the auricle. While there are several reconstructive options available between alloplastic and prosthetic implants, autologous rib cartilage grafts remain the most commonly used treatment modality. Still, this widely used technique is accompanied by significant patient discomfort in a young child and carries additional risks secondary to the traumatic process of rib cartilage extraction, such as pneumothorax and chest wall deformities, and the final esthetic results may not be ideal. To circumvent these limitations, tissue engineering approaches have been used to create a realistic-looking ear that mirrors the complex anatomy of the normal ear. This article reviews the biochemical and biomechanical properties of human auricular cartilage as they relate to design criteria. In addition, a variety of cell sources, biocompatible scaffolds, scaffold-free techniques, and mechanical and biological stimuli are discussed. This review aims to identify knowledge gaps in the literature related to auricular cartilage characteristics and make recommendations to drive the field of auricular tissue engineering.

Key Words: Auricular cartilage, biochemical properties, mechanics, reconstruction, tissue engineering

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Congenital auricular anomalies may occur as an isolated condition or as part of a syndrome, such as hemifacial microsomia or Treacher-Collins syndrome.[1] Although most cases of congenital auricular anomalies are sporadic, hereditary cases have also been described.[2] The most recognized ear congenital anomalies are microtia and anotia. Microtia ("small ear") is a defect in which the external ear is underdeveloped and malformed and only a small part of the pinna is present.[3] Anotia ("no ear"), while rare, is the most severe type of microtia, which involves the complete absence of the pinna and the narrowing or absence of the auditory canal.

The overall prevalence of microtia varies between 0.83 and 17.4 per 10,000 live births, depending on the population studied.[3] The prevalence is nearly 1.2 times higher in males, Asians, and Hispanics.[4] Its inheritance is multifactorial, with a recurrence risk of 5.7%.[5] Most cases are unilateral, and the right side is typically affected.[6] Beyond the ear's apparent visual deformity, children with microtia often experience hearing loss due to the closure or absence of the external auditory canal, which can hamper speech development. In addition, microtia patients exhibit considerable psychosocial consequences, including depression, interpersonal sensitivity, social difficulties, hostility, and aggression.[7] Together, these factors necessitate the treatment of microtia with an interdisciplinary team composed of craniofacial surgeons, psychiatrists, speech therapists, and counselors for the patients and their parents.[8,9]

Despite the evolution of current reconstructive options, the intricacies of the auricular anatomy make it challenging to achieve a satisfactory esthetic outcome. Certainly, the advent of tissue engineering has furthered efforts toward fabricating cartilaginous frameworks tailored to individual patient needs. However, several obstacles remain, including the need for high chondrogenic cell quantities, navigating the nuances of resistance to deformation forces, and troubleshooting sustainable framework longevity. Thus, before embarking on a potential novel solution to auricular framework creation, the biochemical and biomechanical properties of human auricular cartilage need to be clearly defined.

This review outlines the aforementioned properties of the human ear and discusses current techniques and the role of tissue engineering in auricular cartilage reconstruction. Together, this information will enable us to improve the design criteria for an auricular construct that is well-suited for both structural and esthetic needs.

We searched PubMed and Google Scholar databases using the following keywords: "auricular," "reconstruction," "cartilage," "anatomy," "biochemical," mechanical," "tissue engineering," "graft," and "scaffold." We grouped our findings according to anatomy, biochemical content, mechanics, and current methods of repair and reconstruction. In addition, we identified gaps in the literature and suggested potential areas for further investigation to improve auricular cartilage engineering as it relates to the treatment of microtia.

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ANATOMY

The ear can be divided into the external (outer), middle, and inner ear. The external ear (auricle or pinna) is a single piece of elastic cartilage with an elaborate and complex architecture.[10] It resides between 2 layers of perichondrium and is covered by hairless skin.[2] The different parts of the external ear are as follows: 1. Helix (HE); 2. Lobule (LO); 3. Antihelix (AH); 4. Antitragus (AT); 5. Concha (CO); 6. Scapha or scaphoid fossa (SC); and 7. Triangular fossa (TF) (as demonstrated in Fig. 1A). The LO is the only part that is devoid of cartilage and is made up of connective tissue (Fig. 1B).

In utero, mesenchymal proliferation guides the development of auricular structures.[11] Specifically, these proliferations, also known as auricular hillocks, form on the first and second pharyngeal arches. The first pharyngeal arch further divides and folds to form the TR, HR, and cymba concha, while the second pharyngeal arch forms the cavum concha, AH, and AT. The cartilage is then covered by ectodermal-derived epithelium to form a recognizable ear structure as early as the 18th gestational week.[12,13] However, the process of auricular cartilage folding varies for each individual. This variability renders the developing cartilage vulnerable to exertional forces in an unpredictable manner. The AH, HE, and SC are the least rigid, most variable, and highly significant in terms of esthetics.[2] Moreover, in conjunction with the TF and AT, these auricular components are formed from the free ear fold, which makes them malleable and most susceptible to deformational forces in utero and/or ex utero.[2]

TISSUE ENGINEERING

To overcome the limitations associated with autologous and alloplastic reconstructive options while also minimizing surgeon-specific inconsistencies in structural esthetics, prefabricated autologous cartilaginous frameworks have been proposed. The concept of prefabrication was introduced in the 1940s[14] when costal cartilage was harvested, diced, and placed in an ear-shaped metal mold. The mold was banked in the patient's abdomen for months, and the cartilage pieces were eventually joined together by fibrosis. Unfortunately, the structural outcomes of this technique were highly variable because scar tissue contraction distorted the framework.

More recently, tissue engineering techniques have led to expanding autologous chondrocytes in vitro and subsequently seeding them onto various biological or synthetic scaffolds. However, culturing chondrocytes is not ideal due to their limited capacity to proliferate in culture and their tendency to lose their chondrogenic potential with subsequent passages.[15,16]

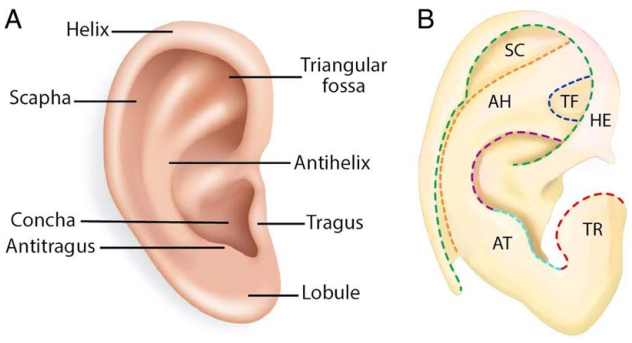

FIGURE 1. Ear anatomy. (A) The anterior surface of the auricle. (B) The anterior surface of the auricular cartilage. AH indicates, antihelix; AT, antitragus; HE, helix; SC, scapha; TF, triangular fossa; TR, tragus.

Consequently, to bolster chondrogenic potential, the addition of mesenchymal stem cells (MSCs) and other biofactors has been investigated.[17–24] These types of 3-dimensional (3D) cartilaginous frameworks have been placed under the loose skin on the dorsum of mice to test survival and maintenance of shape.[25] However, the loose skin of mice does not mirror the conditions encountered in human auricular reconstruction, where the skin in the mastoid region is tightly adherent to the underlying bone or the skin-grafted muscle flap, exerting restrictive forces under which a weak construct can deform. Thus, the successful creation of a tissue-engineered ear requires a strong, yet flexible, framework that can withstand the mechanical disruptions encountered by a normal ear. For this purpose, it is necessary to understand the mechanical, biochemical, and structural properties of auricular elastic cartilage to establish design criteria while also lending mindful consideration to the preconditions of a surgeon.

Mechanical Properties

Auricular cartilage has viscoelastic properties and is primarily composed of collagen and elastin fibers that encompass a proteoglycan gel.[26,27] This structural composition allows the cartilage to retain water, which translates into an elastic response to load. Elastin is the major contributor to the intrinsic mechanical properties of the auricular cartilage. It promotes stiffness at equilibrium and allows the ear to be flexible, maintaining its shape against the micromechanical forces exerted during an individual's lifetime and postsurgery.[28]

To study the microdeformation characteristics of the human auricular cartilage, its mechanical properties through tensile and compressive testing have been characterized and are summarized in the following sections:

Thickness

Various studies report the thickness of auricular cartilage across different anatomic regions and ages (summarized in Supplemental Table 1, Supplemental Digital Content 1, http://links.lww.com/SCS/F904). Griffin and colleagues divided 15 human cadaver auricular cartilage samples into 5 anatomic regions, namely the CO, TR, AT, HE, and AH. They used electronic calipers to measure the thickness of each region and reported that the all HE and AH were significantly thinner than the CO, TR, and AT ($P < 0.01$).[29] Nimeskern et al[30] divided the auricular cartilage into 6 regions (CO, TR, AT, HE, AH, and SC) and noted the AT was the thickest, with little variation among the other regions. They also reported that the older adults (age 50 years or older) had thicker cartilage in comparison to the younger adults (age: 20–34 years).

Chui et al,[31] on the other hand, compared the thickness of human conchal cartilage with that of other species. The tissues from rats, rabbits, and humans were significantly thinner than those from pigs and cows ($P = 0.001$). The cartilage thickness observed in the rat specimens was also significantly thinner than the human specimens ($P = 0.015$). Overall, the thickness of the human samples was higher than that of the rats/rabbits but lower than that of the pigs/cows.[31]

Compressive Properties

The compressive properties of cartilage can be tested using a wide variety of methods. In general, compression indentation techniques and atomic force microscopy have been used to measure Young's modulus and stress-relaxation rates of human auricular cartilage (Supplemental Table 2, Supplemental Digital Content 1, http://links.lww.com/SCS/F904). The testing conditions greatly affect the mechanical properties measured. For example, AFM gives a highly localized measurement of surface

stiffness (hardness), whereas gross compressive indentation gives a bulk property reflective of the whole tissue.[32]

Bos et al[33] used a commercial nanoindenter (Piuma; Optics11, Amsterdam, The Netherlands), capable of applying forces ranging from 0.1 μN to 7.5 mN at indentation depths ranging from 1 to 17 μm. Twenty conchal biopsies were punched out from 10 human auricular cartilage samples, and each sample was indented 10 times at the same anatomic location. The resulting stress-strain curves were analyzed and produced an average compressive Young's modulus of 1.14 ± 0.71 MPa, with considerable variation in stiffness between donors.[33]

Griffin et al,[29] on the other hand, used a Mach-1 material testing device (Biomomentum, Canada), with a 1 kg load cell. Initially, they divided the auricular cartilage into 14 anatomic regions. No significant differences were elucidated between the different parts, with an average overall compressive Young's elastic modulus of 1.66 ± 0.63 MPa, which suggested that auricular cartilage has homogenous compressive properties. However, after reanalyzing the data, the authors delineated a 5-point map, which revealed that the CO (2.08 ± 0.70 MPa) had a significantly greater Young's elastic modulus than the HE (1.41 ± 0.67 MPa) ($P < 0.01$), and a higher rate of loading than the AH in compression ($P < 0.05$). The Young's elastic moduli of the AH, TR, and AT were 1.71 ± 0.63 MPa, 1.67 ± 0.61 MPa, and 1.79 ± 0.56 MPa, respectively. These findings demonstrate that ultrastructural variances account for differences in compressive mechanical properties.[29] The authors also calculated the final stress-relaxation rates for all 5 structural components of the auricular cartilage.[29] The similarities in this parameter among all the regions conveyed that the entire ear could reach a similar load equilibrium over 15 minutes. Moreover, the final absolute relaxation was also similar among the 5 regions, indicating that all parts of the auricular cartilage relax to a similar final stress level.[29]

Chiu et al[31] used the same device to test 5 human pediatric CO cartilage samples and compared them with auricular cartilage samples from rabbits, cows, pigs, and rats. A double compressive indentation method with plane-ended indenters (diameters: 1 and 2 mm), was used to perform indentations at a rate of 10% strain/s to a maximum of 10% strain. There were no significant differences between the mechanical properties of human auricular cartilage and those of other species. The bulk moduli and Young's moduli were higher than rat/rabbit but lower than pig/cow.[31]

Lastly, Pappa et al[26] utilized atomic force microscopy (MFP-3DBio; Asylum Research, Santa Barbara, CA) and compression testing (Bose ElectroForce 3100, Framingham, MA) to determine the elastic moduli and stress-relaxation rates of cartilage samples from various ages of patients from the normal conchal bowl, microtia ears, and preauricular tags. Costal cartilage was also characterized because it is used in autologous reconstruction. All the native samples were compared with tissue-engineered (TE) cartilage generated from umbilical cord MSCs to identify the best candidate for reconstruction purposes. The authors reported that the elastic modulus increased with increasing age in all the native cartilage samples, except the preauricular tags, and it was the highest in the costal cartilage (361 ± 372 kPa), followed by microtia, preauricular appendages, normal conchal cartilage (31.8 ± 18 kPa), and the TE pellet.[26] A similar pattern was observed in the stress-relaxation rates. The highest value was seen in costal cartilage (64.7 kPa/s), followed by microtia, preauricular, and conchal cartilage (15.1 kPa/s). The TE pellet exhibited the lowest value (7.6 ± 1.1 kPa/s).[26]

The geometric stiffness of the whole ear is another mechanical parameter reported in the literature. Zopf et al[34] used an MTS Alliance RT/30 testing machine, equipped with a 500 N load cell, to apply load at a constant displacement rate of 10 mm/min to the entire, intact ear. Nine fresh human auricles from 7 donors and porcine ears from 8 adult pigs were tested in a helix-down position. The stiffness of the human ear was calculated to be 0.194 ± 0.202 N/mm in the linear region between 2 and 4 mm of displacement. Moreover, a nonlinear strain-stiffening elastic behavior characteristic of other physiological soft tissues was observed in both human and porcine ears. The latter, however, was found to be more compliant.[34]

Another study outlined the instantaneous modulus (E_{in}), equilibrium modulus (E_{eq}), and maximum stress (σ_{max}) values of human auricular cartilage. Nimeskern et al[30] tested 15 male and 12 female fresh cartilage samples from children (age below 20 years), young adults (age 20–34 years), middle adults (age 35–49 years), and older adults (age 50 years or older). The E_{in}, E_{eq}, and σ_{max} values were not significantly different among the age groups and genders. In contrast, regional variation patterns across the auricle were observed for E_{in}, E_{eq}, and σ_{max}, where the HE demonstrated the lowest values and the AT the highest. This was significantly different from all the regions except the TR.[30] The authors also estimated the viscoelastic relaxation by measuring the relaxation half-life ($t_{1/2}$) after the first strain application.[30] The $t_{1/2}$ pattern observed showed a slower relaxation in the HE and AH compared with the other 4 anatomic regions of the auricle.[30]

Supplemental Table 2, Supplemental Digital Content 1, http://links.lww.com/SCS/F904 outline the different compressive properties of human auricular cartilage samples stratified by anatomic regions.

Tensile Properties

When evaluating the tensile properties of auricular cartilage, it is important to consider the following 3 parameters: ultimate tensile strength (UTS), stiffness, and resilience.[35] UTS is the maximum tensile stress a material can sustain, after which it fractures. The stiffness of a material corresponds to its Young's modulus in tension and defines the ease of elongation in response to incremental loads.[35,36] Resilience is the energy absorbed by a material during elastic deformation, and it is a measure of how tough a material is, that is, the energy required to break it. These values are calculated using stress-strain graphs generated after a material experiences a tensile load. Stress is the load on the sample normalized to the cross-sectional area, and strain is the resultant elongation normalized to the original length of the specimen.[35] Supplemental Table 3, Supplemental Digital Content 1, http://links.lww.com/SCS/F904 highlight the different experimental setups used to measure the tensile properties of human auricular cartilage samples by various authors.

Zahnert et al[36] obtained cartilage samples from 2 anatomic regions (CO and TR) of 10 fresh human cadavers. The authors found a mean tensile Young's modulus of 3.4 N/mm^2 for conchal cartilage and of 2.8 N/mm^2 for tragal cartilage. Thereafter, Park et al[35] calculated all 3 parameters of tensile properties in 8 specimens of human auricular cartilage (4 males, 4 females). The instantaneous load (kg) and displacement data (inches) was used to generate a stress-strain graph, which showed a UTS of 2.18 MPa, tensile stiffness of 5.11 MPa, and resilience of 0.42 J/m^3[35]. Finally, Nayyer et al[37] tested human auricular cartilage samples (region not specified). The samples showed a maximum tensile strength of 2.02 ± 0.25 MPa, a Young's modulus in tension of 5.02 ± 0.04 MPa, and a $40.62\% \pm 28\%$ elongation at break.[37]

Mechanical Properties Summary

Auricular cartilage has varying degrees of thickness, and the high degree of ultrastructural variations accounts for the differing compressive mechanics. Studies to date reveal that there is a need for optimization of specimen geometry, preparation methods, and testing conditions to understand the mechanical characterization of auricular cartilage. Hence, the suitability of testing methods should be analyzed based on the reproducibility of the results. For these reasons, it is important to re-explore the mechanical properties of human auricular cartilage and establish the techniques/devices that are most reliable and reproducible.

Biochemical Content

The physical characteristics of cartilage, including its viscoelasticity, tensile strength, and compressive strength, depend on the cartilage composition and the amount of collagen, elastin, and proteoglycans in the extracellular matrix (ECM). One study, in particular, demonstrated that the DNA, sulfated glycosaminoglycan (sGAG), and elastin content correlate significantly with the E_{in}, σ_{max}, and E_{eq}.[30] Thus, in order to curate the perfect TE cartilage for auricular reconstruction, the biochemical composition and microstructure of auricular cartilage have been studied using bioassays and histologic examinations.[38] Real-time polymerase chain reaction (RT-PCR) techniques have also been used to quantify gene expression profiles. This information is relevant to the different anatomic parts of the auricle and analyzes differences in age, sex, and conditions, such as microtia. Because a combination of biochemical composition and local tissue morphology is responsible for the regional variations observed in the mechanics of auricular cartilage, we have grouped these findings based on the analyses described in the literature (Supplemental Table 4, Supplemental Digital Content 1, http://links.lww.com/SCS/F904).

Microstructure and Tissue Organization

The principal cells of cartilage, chondrocytes, reside within lacunae.[31] The lacunae are surrounded by an abundant, compact ECM composed of elastin, collagen, and sGAGs. Elastin is present in all cartilage components of the auricle, both intracellularly and extracellularly, and appears as weakly stained thin fibers in the ECM.[29,31] The elastic fibers form a dense lattice network around the individual chondrocytes.[39]

Griffin et al[29] report that the auricle is homogeneous in chondrocyte morphology, ECM, and elastin content. They observed evenly distributed chondrocytes with similar morphologies across all anatomic regions of auricular cartilage.[29] However, zonal differences do exist. Melgarejo-Ramírez[40] observed a change in the shape of the chondrocytes as they approached the perichondrium. Chondrocytes in the peripheral zones appear flatter and closer together in comparison to the central zones.[40] The matrix is also more compact near the perichondrium.[40]

In contrast, Kana and colleagues not only observed zonal but regional differences as well, which contradicted Griffin's findings. For example, they noted that the external surface in an area of concavity (SC) was less cellular, with oval-shaped chondrocytes and a higher density of elastic fibers interspersed between the cells.[39] In contrast, the external surface of the convex side (HE, AH) housed an increased number of chondrocytes, which were more elongated and fusiform in shape and were surrounded by a lower concentration of elastic fibers.[39] Interestingly, in areas devoid of undulating topography, the density and shape of the chondrocytes in both peripheral zones were similar. Overall, the auricular cartilage showed a tri-lamellar structure, with a greater concentration of elastic fibers in the central lamella than both peripheral zones.[39]

Furthermore, examining samples from different age groups under light microscopy has also revealed differences in the number of chondrocytes. Cartilage from younger donors has an increased quantity of chondrocytes, and the elastic fibers form a continuous thick layer. In older donors, fewer chondrocytes are present, with larger vacuoles and fragmented heterogeneous elastic fibers, with multiple branches.[41] However, despite having larger lacunae, the aged human cartilage has a higher Young's modulus compared with their younger counterparts. This may be due to the age-related variations in the ECM components leading to differing cartilage mechanics, which are discussed in the following sections.

In addition to normal age-related changes, researchers have also studied tissue architecture under pathologic conditions, such as microtia.[40] Surprisingly, histologic analyses reveal that despite the external abnormality seen in microtia, the internal tissue organization is similar to that of healthy auricular cartilage.[40]

Elastin

Elastin is the principal protein present in elastic cartilage. Elastin fibers can be stretched by ~200% of their resting length without deforming.[27] Nimeskern et al[30] measured elastin and normalized the values to sample wet mass (Supplemental Table 4, Supplemental Digital Content 1, http://links.lww.com/SCS/F904). They found that auricular cartilage contained a large amount of elastin, with > 15% elastin content per sample wet mass. However, overall, there were no significant regional differences based on age or harvesting location ($P > 0.05$).[30] Pappa and colleagues analyzed both the elastin and fibrillin (a glycoprotein that binds elastin) content in cartilage samples from normal conchal bowls, preauricular appendages, and microtia patients. They then compared them to TE cartilage generated from umbilical cord MSCs.[42] All the native auricular cartilage samples showed similar elastin and fibrillin staining. In contrast, the costal cartilage did not stain for elastin, and the fibrillin staining was weak. The TE pellet had less elastin but a comparable fibrillin content. Moreover, it demonstrated the presence of fibrillin III, which reflects the immaturity of the TE cartilage. Dahl et al[43] also analyzed pediatric microtia cartilage, pediatric preauricular appendages, and normal adult auricular cartilage and observed similar levels and distribution of elastin in all the samples.

Collagen analysis

Collagen is a fibrous protein arranged in parallel fibers that are attached at various locations. This arrangement reflects the tensile strength of cartilage.[27] There are 16 types of collagen.[44] However, collagen II is the major matrix protein in cartilage.[45] Nimeskern and colleagues estimated collagen content by measuring hydroxyproline (HYP).[30] No regional variations were observed, but older adults had significantly lower HYP levels.

As such, Dahl et al[43] revealed the expression of collagens I, II, and X in human auricular cartilage samples through a histologic examination. On gross inspection, collagen II was expressed at a much lower level in the microtia samples (288 cells/mm^2 in microtia, 652 cells/mm^2 in pediatric preauricular tags, and 681 cells/mm^2 in adult conchal bowl). An image analysis demonstrated that collagens I and X were expressed at similar levels in each of the auricular cartilage samples.[43] These findings were corroborated by Pappa et al,[42] who reported similar collagen I, II, and X levels in normal conchal cartilage and

preauricular tags. In microtia samples, the quantities of collagen I and X were similar to the aforementioned cartilages. However, collagen II levels were lower. Interestingly, the presence of calcium was also noted in microtia cartilage, whereas it was absent in all the other samples. Costal cartilage exhibited decreased quantities of all 3 collagen types compared with the auricular samples.[42]

Collagen X is specific to cartilage and is produced by terminally differentiated chondrocytes.[46] It plays a role in matrix mineralization and endochondral ossification and hence serves as a marker for hypertrophic, calcified cartilage.[47] In the absence of any genetic variations, the presence of collagen X in normal pediatric auricular cartilage appears out of place since the ear is a nonmineralizing connective tissue.[48] This property of auricular cartilage is surprising and raises the question of whether collagen X is present in all types of elastic cartilage or if it is specific to the ear cartilage. Animal studies have identified its absence in the epiglottis, which is also composed of elastic cartilage.[49] In contrast, nasal and tracheal cartilage synthesize type X collagen, which persists within the ECM without mineralizing.[49,50] Further elucidation of collagen X's role is required to establish its importance in maintaining the structural integrity of various cartilaginous structures and allow researchers to manipulate its ratios to achieve the perfect tissue-engineered construct.

Glycosaminoglycan (GAG) analysis

Glycosaminoglycans are highly polar, negatively charged polysaccharide compounds present in the ground substance of elastic cartilage that attracts water.[51] There are several types of GAGs, but the most abundant are chondroitin-4 and chondroitin-6 sulfate.[52] Heparin sulfate and chondroitin sulfate are both involved in different stages of elastic fiber development.[53,54] Moreover, hyaluronate and proteoglycan aggregates also co-localize with elastic fibers, without any zonal differences throughout the substance of the auricular cartilage.[55,56] GAGs interact with other compounds, including collagen, laminin, and fibronectin, to form connections and provide structural support. Thus, the quantity of GAGs in auricular cartilage reflects the compressive properties of the connective tissue framework.[51]

Nimeskern and colleagues demonstrated that older adults demonstrated thicker cartilage and lower sGAG (sulfated GAG) levels, suggesting age-dependent differences (Supplemental Table 4, Supplemental Digital Content 1, http://links.lww.com/SCS/F904). Auricular cartilage also displays half the amount of sGAGs compared with nasal septal cartilage. This corresponds to the significantly lower strength, stiffness, and sGAG content and the significantly higher relaxation time and DNA content in auricular cartilage.[30] In addition, regional differences are apparent. For example, sGAGs are more abundant in the AT and TR than the HE. This correlates with the mechanical parameters because the AT is the stiffest, and the HE is the softest when measured mechanically. Furthermore, Pappa et al[42] demonstrate similar levels of GAGs in all native cartilage samples and a lower quantity in a TE pellet.

Due to the involvement of GAGs in the formation of elastic fibers and their interactions with the ECM, we believe that the GAG-matrix binding plays a functional role in the stiffness of auricular cartilage. This hypothesis was tested by Nimeskern et al,[28] when they attempted to remove the sGAGs and found it difficult to completely deplete the sGAGs without affecting the elastin content. This temporal interaction between the 2 components of auricular cartilage requires further exploration from a structural perspective on a larger scale.

DNA Content

Nimeskern et al[30] revealed regional variations in DNA content in the auricular cartilage. The SC had the highest content, and the AT and the TR had the lowest DNA content (Supplemental Table 5, Supplemental Digital Content 1, http://links.lww.com/SCS/F904). In contrast, no significant differences were found between the various age groups or males and females. While this data might signify that postbirth, aging does not affect the proliferative capacity of the chondrocytes, the Ki-67 staining (a marker for proliferation) in adult and 22-week aborted fetal auricular cartilages demonstrate that aging results in a significant prolongation of the cell cycle resulting in an extremely low proliferative activity.[39]

Biochemical Content Summary

The biochemical content of the auricular cartilage influences its mechanical properties. The GAG and collagen content correlate with the stiffness and strength of the cartilage. These differences are reflected in varying degrees of GAGs in the HE, TR, and TR, which corresponds to the variation in mechanical characteristics in these anatomic locations. As humans age, the thickness of the auricular cartilage increases, which is reflected in the fragmentation and heterogeneity observed in elastic fibers, along with a reduction of GAG and collagen content. Moreover, the chondrocytes, within the matter of the cartilage, reside in small lacunae, which enlarge with age.[41] Microtia cartilage demonstrates decreased functional elasticity compared with normal auricular cartilage, which is evidently due to the increased calcium content. As discussed previously, in addition to the compressive properties, the biochemical composition of preauricular cartilage remnants is similar to conchal cartilage and hence, may provide the best source for TE cartilage. Overall, there is a paucity of quantitative data characterizing the biochemical properties of human auricular cartilage and contradicting results on its microstructural organization. Hence, future work should focus on quantitatively analyzing the ECM components on a larger scale and studying the general morphology and cellular architecture in detail.

Scaffold and Scaffold-Free Techniques for TE Cartilage

Scaffolds provide a stable 3D framework that host cells and other biological molecules that secrete ECM and regenerate functional tissues.[57] In addition to providing the cells with an appropriate microenvironment that mimics native tissue, an ideal scaffold must have the following properties: 1) adequate pore size; 2) a large surface area; 3) a rate of scaffold degradation that is in equilibrium with the rate of tissue regeneration; 4) suitable mechanical strength to maintain the predesigned structure; 5) biocompatibility; and 5) a positive interaction with the cells, including cellular adhesion, migration, proliferation, and differentiation to achieve optimal biomaterial-tissue integration.[57,58]

Recently, 3D bioprinting technology has made it possible to generate intricate scaffolds of a predetermined shape, such as an ear.[59] The contralateral ear is scanned and measured so that the resultant scaffold precisely mirrors the contours and projections of the normal ear.[60] Despite this feat, scientists continue to struggle to render a scaffold with an adequately large pore size ratio that facilitates deeper migration of cells into the scaffold without compromising its mechanical stability. Moreover, the accuracy of the printing nozzle and the precise size of the bio-ink particles are difficult to control.[61] While the laser-assisted printing technology may be considered a good alternative, the

lower cellular viability compared with other bioprinting methods, along with the time-consuming nature of the process, and the high cost limit its use.[61] Therefore, maintaining this structure in vitro and in vivo has proven challenging.

The choice of material used, whether synthetic or naturally derived, a porous or hydrogel-based polymer, is critical to the success of tissue engineering cartilage. Several biomaterials and their hybrids have been used to generate auricle-shaped constructs (Supplemental Table 5, Supplemental Digital Content 1, http://links.lww.com/SCS/F904).[58,62] Collagen, the most abundant ECM component, is recognized as a promising biomaterial for scaffolds in tissue engineering. The benefits of using collagen as a biological scaffold include its low immunogenicity, porous structure, and favorable biocompatibility. However, collagen scaffolds often lack the rigidity needed for tissue engineering purposes.[63]

Another subtype of naturally occurring scaffold that has been used in cartilage engineering includes decellularized human tissue, such as acellular cartilage matrix (ACM) and allograft adipose matrix (AAM). Jia et al[64] demonstrated that an ACM/gelatin suspension, with a polycaprolactone (PCL) inner core, can be prepared into a porous scaffold using 3D printing. The authors noted a cell seeding efficiency of more than 90%, and after 14 days of growth, they implanted the newly formed cartilage-like tissue into nude mice. Subsequent analyses, 6 and 12 weeks after implantation, showed gradual regeneration of mature cartilage with abundant lacunae and a homogenous ECM distribution. However, little fibrous tissue was seen up until 12 weeks. The DNA, GAG, and collagen contents showed levels equivalent to native cartilage. The constructs also maintained their original shape with good elasticity.[64] However, the initial heterogeneous nature of the cartilage and the presence of fibrous tissue signifies that optimization of the scaffold preparation and further evaluation of the scaffold biosafety are required.

Our laboratory utilized AAM as a disc-like framework to coculture auricular chondrocytes (AuCs) and adipose-derived stem cells (ADSCs) and observed chondrogenesis induction.[65] While our experimental bioscaffold lacked pores, resulting in cells' inability to penetrate the entire AAM disc, it confirmed that AAM preserves the essential components of native ECM, which provided the necessary microenvironment for activating chondrogenesis. In the future, 3D bioprinting AAM into a scaffold will allow us to control the porosity better and establish a construct capable of being fully infiltrated by cells.

As an alternative to natural materials, biodegradable synthetic polymers, such as polycaprolactone (PCL), polyglycolic acid (PGA), and poly-L-lactic acid (PLLA), offer certain advantages for developing scaffolds in tissue engineering. The key benefits include controllable mechanical properties and overall malleability, as these scaffolds can be fabricated into various shapes with different pore sizes. However, synthetic materials may elicit a foreign body response from an immunocompetent host.[66] This is problematic because the inflammation leads to the generation of a fibrous capsule, impeding the integration of the implant into the host tissue. Moreover, long-term exposure to inflammatory cells can lead to unwanted corrosion of the implanted biomaterials and has been shown to negatively impact the seeded cells' ability to regenerate and induce chondrogenesis.[67]

To further improve upon these tissue engineering techniques, composite scaffolds that utilize both biological and synthetic materials have been created. The synthetic component provides mechanical strength, while the naturally occurring substances, such as collagen, mimic the native tissue environment.[68] This technique was utilized by Zhou and colleagues when they used autologous chondrocytes derived from microtia cartilage and seeded them onto a scaffold comprised of a PCL mesh inner core, wrapped with PGA unwoven fibers, and coated with polylactic acid (PLA). The structure was created using a negative mold of the patient's ear, and the biomaterials were cast into the ear-shaped mold. This system created an in vitro environment for generating cartilage, and after 12 weeks, the cultured scaffolds were implanted into humans.[66] However, even after 18 months postimplantation, the PCL inner core was still detectable.[66] Moreover, the study described using a negative mold to generate the ear shape by casting the biomaterials. It would be ideal to directly fabricate the 3D ear shape with the scaffold material and possibly with the cells.

Scaffold-free methods have also been developed to engineer cartilage. These include cell sheet engineering, aggregate engineering, and the self-assembling process. Aggregate engineering is a cell culture technique that provides a 2-dimensional structure for cell-to-cell interactions resulting in free-floating cell aggregates.[69] Cell sheet engineering uses a cultured monolayer subjected to thermal energy and physical manipulations, a process that may or may not use enzymatic cleavage depending upon the mandrel used.[70] With this approach, multiple cell layers are added on top of one another to add depth to the TE framework. In contrast, the self-assembling process helps aggregate chondrocytes independent of external energy, where inherent free energy and cellular tension drive cell-to-cell adhesion, which mimics the mesenchymal condensation seen during native development.[70]

Although these modalities appear promising, they do present limitations. As mentioned before, one of the well-established challenges in tissue engineering cartilage for auricular construction is the incredibly high number of chondrocytes required. Microtia cartilage lacks a sufficient number of normal chondrocytes to meet this demand. In addition, once the cells are in monolayer culture, adequate numbers still cannot be obtained due to the loss of the chondrogenic capacity of the chondrocytes.

To reconcile these challenges, Yanaga et al[71,72] developed a multilayer culture system supplemented with fibroblast growth factor-2 (FGF-2). In this environment dedifferentiated chondrocytes re-differentiate, secrete hyaluronic acid, and eventually form a scaffold in vitro.[71] The multilayer, cultured chondrocytes were injected subcutaneously in the lower abdominal wall of 12 patients. After 6 months, the neocartilage was extracted, sculpted into an auricle, and retransplanted in the mastoid region. Mechanical and histologic analyses revealed similarities to the native ear cartilage, and a 6-year follow-up of 1 patient demonstrated morphologic maintenance without absorption.[71] While this technique bypasses the limitations of implanting synthetic or biological materials, it requires additional surgeries, similar to autologous reconstruction. Taken together, the current constraints associated with scaffold and scaffold-free cartilage engineering necessitate the development of even better techniques that are amenable to ear reconstruction.

Cell Sources

When considering auricular chondrocytes (AuCs) to create a cartilaginous framework, we must consider the underlying pathologic processes that result in microtia. Specifically, it has been suggested that "microtia cartilage is not simply smaller normal cartilage, but cartilage that cannot reach the maturity and organization of healthy auricular cartilage."[73] This hypothesis undoubtedly impacts the choice of ear sample used to harvest the chondrocytes. While Zhou et al[66] successfully implanted a tissue-engineered auricle in 5 patients using microtia chondrocytes, 2 of the 5 ears demonstrated distortion when examined 6 months postoperatively. Interestingly, Dahl et al[43] reported that microtia cartilage contains lower levels of collagen

II. However, a tragal biopsy from one of the tissue-engineered constructs implanted by Zhou et al[66] showed a notable collagen II. These discrepant findings warrant further characterization of microtia cartilage to determine the course of the pathologic signaling once it is transferred onto a scaffold. Likewise, Yanaga and colleagues used microtic chondrocytes as seed cells for clinical auricular reconstruction and monitored 1 patient postoperatively over the course of 6 years. They noted that the neocartilage maintained its structure without absorption, concluding that this was because of the soft, yet viscoelastic character of the newly formed cartilage that resembled native auricular cartilage.[71] Additional long-term patient follow-ups should be carried out to further analyze the structural integrity of the TE framework after the scaffold has degraded.

Alternatively, allogeneic chondrocytes have been hypothesized to be a viable cell source. The avascular nature of auricular cartilage is suggested to make it immunologically privileged, allowing its transplantation into another human.[74] This may be true for transplanted fragments of allogeneic cartilage under physiological conditions, but isolated allogeneic chondrocytes have been known to elicit an immune response.[75] During transplantation, their surface molecules (major histocompatibility complex, MHC) encounter immunocompetent cells, and while they may form cartilage similar to their parent source, it is gradually resorbed by the infiltrating immune cells. In contrast, chondrocytes have also been documented to have immunosuppressive properties by disrupting T-cell function and inhibiting MHC-II expression.[75] These conflicting results are mirrored in the literature, where several studies highlight the possibility of transplanting allogeneic articular chondrocytes[74,76] and others report immune rejection.[77–79]

In light of the considerations mentioned above, the current most practical cell source suited for auricular cartilage engineering reported in the literature remains autologous AuCs derived directly from a patient's microtia ear or, if necessary, from a small biopsy of the patient's unaffected, contralateral conchal bowl.[80] For microtia patients, the typically discarded microtia ear cartilage has the potential to increase the initial chondrocyte population in combination with normal AuCs for cell expansion. AuCs are easily harvested with less donor-site morbidity, produce twice the cell yield per gram, and proliferate faster in a monolayer culture when compared with articular cartilage.[81] One gram of elastic cartilage provides roughly 10 million cells that can be subsequently expanded to generate ~138 million AuCs after just three passages.[82] However, this number is still less than the purported 200 to 250 million cells required to create a full-scale pediatric auricle.[83]

While cell expansion allows us to achieve the desired cell quantities, there remains the challenge that chondrocyte expansion beyond the second passage (P2) results in "dedifferentiation," marked by decreased deposition of chondrogenic matrix materials (ie, type II collagen) and increased fibroblastic behavior (ie, type I collagen deposition).[15,16] The initial few studies that did utilize late-passage human AuCs (HAuCs) were not promising.[15,16,84] But in 2018, Bernstein et al[82] conducted an in vivo experiment in nude mice and discovered that HAuCs expanded through P3, 4, and 5, maintained not only their original cylindrical geometry but also their histologic and biochemical integrity with similar amounts of collagen, proteoglycan, elastin ann hydroxyproline content when compared with native auricular cartilage.

Furthermore, studies have demonstrated that co-culturing P2 expanded cells with freshly isolated chondrocytes over 4 weeks renews their ability to secrete abundant ECM.[85,86] Others have looked into the prospect of growth factors and co-stimulatory molecules as a means to stave off dedifferentiation.[71,85,87] These studies illustrate that the in vitro changes resulting in dedifferentiation are reversible with the right environmental cues. However, their clinical translatability has yet to be validated.

Co-stimulatory cells and molecules

Various cytokines and growth factors have demonstrated the potential to abrogate the dogma of chondrocyte dedifferentiation and enhance the chondrogenic capacity of cells. For instance, pediatric AuCs incubated with insulin and triiodothyronine (T_3) in vitro and with BMP-2 within the atelocollagen hydrogel produce the greatest quantities of GAGs and viable cells compared with other combinations of the 3 stimulatory molecules tested in vitro and within the scaffold.[88] The organization of this model and the timing of the introduction of the growth factors is crucial because insulin and T_3 have a short half-life. Faster degradation causes them to function better in the medium, with frequent medium changes. In contrast, BMP-2 has a half-life of 5 to 6 days, allowing it to function as a stable growth factor in the scaffold.[88] In addition to timing, the selection (and combination) of optimum growth factors has been studied. b-FGF increases the chondrocyte number 5.5-fold in 3 weeks in vitro and produces a higher quantity and quality of tissue in vivo compared with TGF-β.[89] While these results highlight the importance of identifying the optimal concentrations and combinations of growth factors to be applied at distinct time points during chondrogenesis to create a TE construct similar to native auricular cartilage, the literature discussing the effects of various growth factors and proteins on human AuCs is scarce. Future experiments must focus on that direction in order to increase the translatability of these experiments.

In addition to biofactors, the use of stem cells has also been explored to aid in cell expansion. Mesenchymal stem cells (MSCs) are multipotent stem cells with immense chondrogenic potential. Their ability to continuously proliferate and achieve sufficient numbers in vitro may allow for cell growth and ameliorate the issue of dedifferentiation. They are also available in high numbers without any associated ethical concerns, and an increasing number of methods have been identified for their isolation and expansion.[90] Bone marrow mesenchymal stem cells (BMSCs) are the most comprehensively used and investigated MSCs. Nearly 40 studies have implemented MSCs for the clinical repair of cartilage, albeit primarily articular cartilage.[90] Co-culturing MSCs with articular chondrocytes,[24] meniscal fibrochondrocytes,[22,23] and nucleus pulposus cells[21] all resulted in the enhancement of cartilage development. Co-culturing and coimplanting MSCs with AuCs have also been demonstrated with animal cells[19,20] and human-animal cell hybrids.[18,19] In the setting of auricular tissue engineering, Zhang et al[18] cotransplanted microtia chondrocytes with BMSCs (25% MCs and 75% BMSCs) and successfully demonstrated the strong chondroinductive ability of microtia chondrocytes to promote the stable ectopic chondrogenesis of BMSCs in a subcutaneous environment. Moreover, cell labeling showed that BMSCs had the propensity to transform into chondrocyte-like cells secondary to the chondrogenic niche afforded by the cocultured MCs. Most importantly, a human-ear-shaped cartilaginous tissue with a delicate structure and proper elasticity was successfully constructed by seeding the mixed cells (microtia chondrocytes and BMSCs) into a preshaped biodegradable ear-shaped scaffold, followed by 12 weeks of subcutaneous implantation in a nude mouse.

Despite these successes, BMSC harvesting still has several limitations. There is a significant amount of pain and morbidity associated with the bone marrow collection procedure, and the number of harvested bone marrow cells that are MSCs is rela-

tively small (hundreds of cells per ml of marrow).[91] Also, when BMSCs are expanded in vitro, they show signs of senescence.[92] Thus, alternative and comparable sources of MSCs are desirable. Adipose-derived stem cells (ADSCs) are a favorable alternative, as they can be derived from adipose tissue through minimally invasive means, yielding tens of thousands of ADSCs per ml of lipoaspirate.[93] To the best of our knowledge, only one previous coimplantation study combined both AuCs and stem cells of human origin, coimplanting AuCs with ADSCs.[17] AuCs from microtia tissue promoted the chondrogenic differentiation and chondrogenesis of ADSCs by co-grafting in vivo. We confirmed these results in our laboratory by co-culturing AuCs and ADSCs in vitro and examining the gene expression of type II collagen and *SOX9* (key regulators of chondrogenesis).[65] In our efforts, we determined that a 1:9 (AuCs:ADSCs) culture ratio provided an acceptably optimum microenvironment suited for chondrogenesis.[65] Given the substantially lower number of chondrocytes required in our co-culture setting, the utilization of ADSCs may circumvent the need for conventional chondrocyte expansion.[24] These co-culture systems using ADSCs or other MSCs, combined with AuCs, demonstrate advantageous paracrine signaling that may mimic the native cell-cell interactions lost through the enzymatic digestion and processing of the cells.[94] As a result, this new microenvironment is conducive to functionally stable AuCs.[84,87,95,96] Moving forward, more attention should be focused on a means to optimize this microenvironment so that a clinically relevant expansion of chondrocytes can be achieved in a consistent, predictable manner. Figure 2 illustrates the current techniques used in tissue engineering for auricular cartilage.

Mechanical Stimulation

Apart from biological factors, another component of the microenvironment surrounding the chondrocytes affecting their differentiation and proliferation is mechanical loading. Bioreactors capable of transmitting sound waves, microgravity, hydrostatic pressure, compression, and fluid shear, magnetic, electric forces have been created to reproduce the mechanical microenvironment of native cartilage.[97–99] These 3D biomimetic culture systems allow cellular interactions with the application of physical stimulation under optimal levels of oxygen, carbon dioxide, pH, temperature, and growth factors to enhance cell proliferation and matrix deposition.[100,101]

Static compression inhibits, and dynamic compression promotes biochemical anabolism.[102] Chung et al[103] applied a 5% static compression, followed by a dynamic axial strain of 10% at 1 Hz after achieving equilibrium. They also tested higher frequencies (3 Hz) and larger amplitudes (15% dynamic strain) on both in vitro-cultured AuCs and cell-seeded hyaluronic acid hydrogels. Collagen II and aggrecan increased (1.3-fold and 1.4-fold, respectively) in the AuC-constructs after 5 days of consecutive loading, whereas the expression of collagen I was downregulated. However, the increase in ECM components was more robust for the articular cartilage after mechanical compression.[103] These results highlight the inherent differences in the physiological characteristics of different types of chondrocytes, which is expected because articular cartilage undergoes massive bending and buckling forces. In contrast, auricular cartilage is a nonload-bearing structure. The literature is densely populated with studies reporting the effects of various forms of physical stimulation on articular chondrocytes, but the tissue engineering field related to auricular cartilage is still relatively novel. As such, the magnitude of various types of mechanical, electrical, or magnetic forces needs to be adjusted to levels suitable for auricular chondrocytes to promote the development of functional tissue. Moreover, standardized methods and outcome measures of mechanical loading systems are required to facilitate comparisons between various experiments.

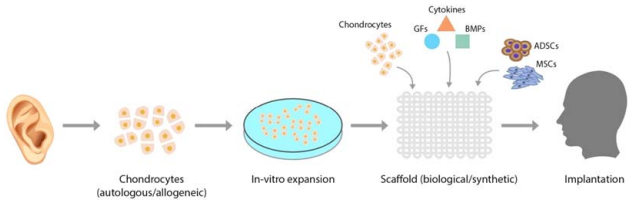

FIGURE 2. Current technique used in tissue engineering auricular cartilage. Autologous or allogeneic chondrocytes are isolated from auricular cartilage and subsequently expanded in vitro. Once expanded, they are added onto a biological or synthetic scaffold, with or without co-stimulatory molecules and/or stem cells (ADSCs or MSCs). Together, they form elastic cartilage, which is ultimately implanted into the patient.

CONCLUSION AND FUTURE DIRECTIONS

This review sheds light on the need for cartilage tissue engineering for auricular reconstruction and outlines the importance of establishing design criteria to achieve biomimetic auricular cartilage. We also provided insight into gaps in the literature pertaining to the biological and mechanical characteristics of auricular cartilage and the current techniques used to engineer elastic cartilage. We believe the first step is to perform an in-depth assessment of the properties inherent to auricular cartilage, most notably collagen X and the GAG-elastin interactions, which may be responsible for its native characteristics. Large-scale studies that include various anatomic regions, congenital pathologies, ages, sexes, and ethnicities must be performed to understand the diversity seen in individual patients.

Several framework designs have been proposed that use a variety of scaffolds, cellular components, and co-stimulatory molecules. The use of AAM in our lab has proven to be chondroinductive in the presence of AuCs and ADSCs. Thus, 3D printing AAM or other ECM scaffolds might be a means to achieve an auricle-shaped construct that mimics the native ear. Since AAM originates from cadaveric donor tissues using standardized and controlled methods, there is also an opportunity for large-scale manufacturing to create an off-the-shelf scaffold. In addition to the scaffolding techniques, the scaffold-free tissue engineering approach also appears promising. However, because the shape of the resultant ear will still be dependent on the surgeon's artistic competency, future studies should focus on eliminating the esthetic variability by combining this approach with a mold using computer-aided design software.

The ideal choice of cells for cartilage generation is understandably normal auricular chondrocytes, but it is imperative to troubleshoot alternative solutions due to their low availability. For instance, continued work on the co-transplantation of accessible sources of MSCs, such as ADSCs, as well as an in-depth review examining a gamut of possible co-stimulatory molecules and their optimal concentrations and combinations in relation to chondrogenesis, should be prioritized. Alternatively, the potential for utilizing allogeneic chondrocytes should be explored. A thorough comprehension of phenotypic stability, function, and antigenicity is required to implement allogeneic tissue-engineered neocartilage successfully. As in the case of rhinoplasty, irradiated and decellularized auricular cartilage can also be tested for auricular reconstruction.

While it is vital to achieve a framework that is biologically, structurally, and mechanically similar to native cartilage, we must

keep in mind that there are restrictive and jarring forces (often related to the nature of the reconstructed skin or muscle envelope) exerted upon a transplanted auricular cartilage framework that can potentially distort the 3-dimensional complex. Hence, the tissue-engineered framework, in addition to being flexible, must have greater tensile and compressive strengths compared with the native structure. For this reason, future work should be directed toward exploring the optimal biological molecules for cross-linking purposes. Various types and magnitudes of mechanical stimulation under favorable conditions must also be tested to mold the TE constructs. The ability to control the mechanical and biochemical properties of the TE cartilage will allow us to achieve a high degree of customization.

Taken together, we are optimistic that advances in tissue engineering and regenerative medicine will allow for an engineered auricle that mimics the natural auricle. Collaborations across all disciplines are crucial to achieving this objective. In addition to the involvement of biomedical engineers, surgeons, and researchers, it might be beneficial to involve patients or their guardians in the dialog relating to their concerns and expectations, which may potentially alter the design process to achieve a more satisfactory outcome.

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REFERENCES

1. Luquetti DV, Leoncini E, Mastroiacovo P. Microtia-anotia: a global review of prevalence rates. *Birth Defects Res A Clin Mol Teratol* 2011;91:813–822
2. Porter CJW, Tan ST. Congenital auricular anomalies: topographic anatomy, embryology, classification, and treatment strategies. *Plast Reconstr Surg* 2005;115:1701–1712
3. Luquetti DV, Heike CL, Hing AV, et al. Microtia: epidemiology and genetics. *Am J Med Genet A* 2012;158A:124–139
4. Cabrejo R, Persing J, Alperovich M. Epidemiologic assessment of microtia in over 23 million consecutive United States births. *J Craniofac Surg* 2019;30:342–346
5. Takahashi H, Maeda K. Survey of familial occurrence in 171 microtia cases. *Jpn J Plast Surg* 1982;15:310–319
6. Brent B. Auricular repair with autogenous rib cartilage grafts: two decades of experience with 600 cases. *Plast Reconstr Surg* 1992;90: 355–374; discussion 375–376
7. Du JM, Zhuang HX, Chai JK, et al. Psychological status of congenital microtia patients and relative influential factors: analysis of 410 cases. *Zhonghua Yi Xue Za Zhi* 2007;87:383–387
8. Horlock N, Vögelin E, Bradbury ET, et al. Psychosocial outcome of patients after ear reconstruction: a retrospective study of 62 patients. *Ann Plast Surg* 2005;54:517–524
9. Steffen A, Meyer Zu Natrup C, König IR, et al. Analysis of psychosocial changes following ear reconstruction with rib cartilage-development of a short test. *Laryngo-rhino-otologie* 2008;88:247–252
10. Szymanski A, Geiger Z. Anatomy, head and neck, ear. 2017. Accessed January 30, 2022. https://europepmc.org/article/nbk/nbk470359
11. Helwany M, Tadi P. Embryology, Ear. *StatPearls*. Treasure Island, FL: StatPearls Publishing; 2020
12. Anthwal N, Thompson H. The development of the mammalian outer and middle ear. *J Anat* 2016;228:217–232
13. Development of the ears. *Reference Module in Biomedical Sciences*. Elsevier; 2014
14. Peer LA. Reconstruction of the auricle with diced cartilage grafts in a vitallium ear mold. *Plast Reconstr Surg* 1948;3:653–666.
15. Benya P. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell* 1982;30: 215–224
16. Benya PD, Padilla SR, Nimni ME. Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. *Cell* 1978;15:1313–1321
17. Cai Z, Pan B, Jiang H, et al. Chondrogenesis of human adipose-derived stem cells by in vivo co-graft with auricular chondrocytes from microtia. *Aesth Plast Surg* 2015;39:431–439
18. Zhang L, He A, Yin Z, et al. Regeneration of human-ear-shaped cartilage by co-culturing human microtia chondrocytes with BMSCs. *Biomaterials* 2014;35:4878–4887
19. Kang N, Liu X, Guan Y, et al. Effects of co-culturing BMSCs and auricular chondrocytes on the elastic modulus and hypertrophy of tissue engineered cartilage. *Biomaterials* 2012;33: 4535–4544
20. Morrison KA, Cohen BP, Asanbe O, et al. Optimizing cell sourcing for clinical translation of tissue engineered ears. *Biofabrication* 2016; 9:015004.
21. Yamamoto Y, Mochida J, Sakai D, et al. Upregulation of the viability of nucleus pulposus cells by bone marrow-derived stromal cells. *Spine* 2004;29:1508–1514
22. McCorry MC, Puetzer JL, Bonassar LJ. Characterization of mesenchymal stem cells and fibrochondrocytes in three-dimensional co-culture: analysis of cell shape, matrix production, and mechanical performance. *Stem Cell Res Ther* 2016;7.
23. McCorry MC, Bonassar LJ. Fiber development and matrix production in tissue-engineered menisci using bovine mesenchymal stem cells and fibrochondrocytes. *Connect Tissue Res* 2017;58:329–341
24. Meretoja VV, Dahlin RL, Kasper FK, et al. Enhanced chondrogenesis in co-cultures with articular chondrocytes and mesenchymal stem cells. *Biomaterials* 2012;33:6362–6369
25. Cao Y, Vacanti JP, Paige KT, et al. Transplantation of chondrocytes utilizing a polymer-cell construct to produce tissue-engineered cartilage in the shape of a human ear. *Plast Reconstr Surg* 1997;100:297–302; discussion 303–304
26. Pappa AK, Soleimani S, Caballero M, et al. A pilot study comparing mechanical properties of tissue-engineered cartilages and various endogenous cartilages. *Clin Biomech* 2017;50: 105–109
27. Watkins J, Mathieson I. Connective tissues. *Pocket Podiatry Guide* 2009:107–156
28. Nimeskern L, Utomo L, Lehtoviita I, et al. Tissue composition regulates distinct viscoelastic responses in auricular and articular cartilage. *J Biomech* 2016;49:344–352
29. Griffin MF, Premakumar Y, Seifalian AM, et al. Biomechanical characterisation of the human auricular cartilages: implications for tissue engineering. *Ann Biomed Eng* 2016;44:3460–3467
30. Nimeskern L, Pleumeekers MM, Pawson DJ, et al. Mechanical and biochemical mapping of human auricular cartilage for reliable assessment of tissue-engineered constructs. *J Biomech* 2015;48: 1721–1729
31. Chiu LLY, Giardini-Rosa R, Weber JF, et al. Comparisons of auricular cartilage tissues from different species. *Ann Otol Rhinol Laryngol* 2017;126:819–828
32. Yoo L, Reed J, Shin A, et al. Atomic force microscopy determination of Young's modulus of bovine extra-ocular tendon fiber bundles. *J Biomech* 2014;47:1899–1903
33. Bos EJ, Pluemeekers M, Helder M, et al. Structural and mechanical comparison of human ear, alar, and septal cartilage. *Plast Reconstr Surg Glob Open* 2018;6:e1610
34. Zopf DA, Flanagan CL, Nasser HB, et al. Biomechanical evaluation of human and porcine auricular cartilage. *Laryngoscope* 2015;125:E262–E268
35. Park SS, Jin HR, Chi DH, et al. Characteristics of tissue-engineered cartilage from human auricular chondrocytes. *Biomaterials* 2004;25:2363–2369
36. Zahnert T, Hüttenbrink KB, Mürbe D, et al. Experimental investigations of the use of cartilage in tympanic membrane reconstruction. *Am J Otol* 2000;21:322–328
37. Nayyer L, Birchall M, Seifalian AM, et al. Design and development of nanocomposite scaffolds for auricular reconstruction. *Nanomedicine* 2014;10:235–246

38. Sasano Y, Takahashi I, Mizoguchi I, et al. Type X collagen is not localized in hypertrophic or calcified cartilage in the developing rat trachea. *Anat Embryol* 1998;197:399–403
39. Kaňa M, Kaňa R, Povýšil C. New developments in understanding the histological structure of human ear cartilage. *Folia Biol* 2019; 65:256–264
40. Melgarejo-Ramírez Y, Sánchez-Sánchez R, García-López J, et al. Characterization of pediatric microtia cartilage: a reservoir of chondrocytes for auricular reconstruction using tissue engineering strategies. *Cell Tissue Bank* 2016;17:481–489
41. Ito I, Imada M, Ikeda M, et al. A morphological study of age changes in adult human auricular cartilage with special emphasis on elastic fibers. *Laryngoscope* 2001;111:881–886
42. Pappa AK, Caballero M, Dennis RG, et al. Biochemical properties of tissue-engineered cartilage. *J Craniofac Surg* 2014; 25:111–115
43. Dahl JP, Caballero M, Pappa AK, et al. Analysis of human auricular cartilage to guide tissue-engineered nanofiber-based chondrogenesis: implications for microtia reconstruction. *Otolaryngol Head Neck Surg* 2011;145:915–923
44. Lodish, Harvey F. Molecular cell biology. *Macmillan*, 2008. 8th edition. (Section 20.4, The extracellular matrix II: connective tissue) 2008:951–960.
45. Aszódi A, Hunziker EB, Olsen BR, et al. The role of collagen II and cartilage fibril-associated molecules in skeletal development. *Osteoarthritis Cartilage* 2001;9(suppl A):S150–S159
46. Gudmann NS, Karsdal MA. Chapter 10 - Type X Collagen. *Biochemistry of Collagens, Laminins and Elastin*. Academic Press; 2016:73–76
47. Shen G. The role of type X collagen in facilitating and regulating endochondral ossification of articular cartilage. *Orthod Craniofac Res* 2005;8:11–17
48. Chiu LLY, Giardini-Rosa R, Weber JF, et al. Comparisons of Auricular Cartilage Tissues from Different Species. *Annals of Otology, Rhinology & Laryngology* 2017;126:819–828
49. Naumann A, Dennis JE, Awadallah A, et al. Immunochemical and mechanical characterization of cartilage subtypes in rabbit. *J Histochem Cytochem* 2002;50:1049–1058
50. Sasano Y, Takahashi I, Mizoguchi I, et al. Type X collagen is not localized in hypertrophic or calcified cartilage in the developing rat trachea. *Anatomy and embryology* 1998;197:399–403
51. Casale J, Crane JS. Biochemistry, glycosaminoglycans. *StatPearls*. Treasure Island, FL: StatPearls Publishing; 2020
52. Bentley JP, Rokosová B. The metabolic heterogeneity of rabbit ear cartilage chondroitin sulphate. *Biochem J* 1970;116:329–336
53. Cain SA, Baldock C, Gallagher J, et al. Fibrillin-1 interactions with heparin. Implications for microfibril and elastic fiber assembly. *J Biol Chem* 2005;280:30526–30537
54. Wu WJ, Vrhovski B, Weiss AS. Glycosaminoglycans mediate the coacervation of human tropoelastin through dominant charge interactions involving lysine side chains. *J Biol Chem* 1999;274: 21719–21724
55. Wusteman FS, Gillard GC. Hyaluronic acid in elastic cartilage. *Experientia* 1977;33:721–723
56. Baccarani-Contri M, Vincenzi D, Cicchetti F, et al. Immunocytochemical localization of proteoglycans within normal elastin fibers. *Eur J Cell Biol* 1990;53:305–312
57. Ma PX. Scaffolds for tissue fabrication. *Mater Today* 2004;7: 30–40
58. Bichara DA, O'Sullivan NA, Pomerantseva I, et al. The tissue-engineered auricle: past, present, and future. *Tissue Eng Part B Rev* 2012;18:51–61
59. Kačarević Ž, Rider P, Alkildani S, et al. An introduction to 3D bioprinting: possibilities, challenges and future aspects. *Materials* 2018;11:2199.
60. Mussi E, Furferi R, Volpe Y, et al. Ear reconstruction simulation: from handcrafting to 3D printing. *Bioengineering (Basel)* 2019;6.
61. Bishop ES, Mostafa S, Pakvasa M, et al. 3-D bioprinting technologies in tissue engineering and regenerative medicine: current and future trends. *Genes Dis* 2017;4:185–195
62. Kim MS, Kim HK, Kim DW. Cartilage tissue engineering for craniofacial reconstruction. *Arch Plast Surg* 2020;47:392–403
63. Zhao X, Bichara DA, Zhou L, et al. Conditions for seeding and promoting neo-auricular cartilage formation in a fibrous collagen scaffold. *J Craniomaxillofac Surg* 2015;43:382–389
64. Jia L, Zhang Y, Yao L, et al. Regeneration of human-ear-shaped cartilage with acellular cartilage matrix-based biomimetic scaffolds. *Appl Mater Today* 2020;20:100639.
65. Ziegler ME, Sorensen AM, Banyard DA, et al. Improving in vitro cartilage generation by co-culturing adipose-derived stem cells and chondrocytes on an allograft adipose matrix framework. *Plast Reconstr Surg* 2021;147:87–99
66. Zhou G, Jiang H, Yin Z, et al. In vitro regeneration of patient-specific ear-shaped cartilage and its first clinical application for auricular reconstruction. *EBioMedicine* 2018;28:287–302
67. Saleh LS, Bryant SJ. The host response in tissue engineering: crosstalk between immune cells and cell-laden scaffolds. *Curr Opin Biomed Eng* 2018;6:58–65
68. Haaparanta AM, Järvinen E, Cengiz IF, et al. Preparation and characterization of collagen/PLA, chitosan/PLA, and collagen/chitosan/PLA hybrid scaffolds for cartilage tissue engineering. *J Mater Sci Mater Med* 2014;25:1129–1136
69. Welter JF, Solchaga LA, Penick KJ. Simplification of aggregate culture of human mesenchymal stem cells as a chondrogenic screening assay. *Biotechniques* 2007;42:732734–737
70. Lee JK, Link JM, Hu JCY, et al. The self-assembling process and applications in tissue engineering. *Cold Spring Harb Perspect Med* 2017;7:a025668.
71. Yanaga H, Imai K, Koga M, et al. Cell-engineered human elastic chondrocytes regenerate natural scaffold in vitro and neocartilage with neoperichondrium in the human body post-transplantation. *Tissue Eng Part A* 2012;18:2020–2029
72. Yanaga H, Imai K, Koga M, et al. Cell-engineered human elastic chondrocytes regenerate natural scaffold in vitro and neocartilage with neoperichondrium in the human body post-transplantation. *Tissue Eng Part A* 2012;18:2020–2029
73. Zucchelli E, Birchall M, Bulstrode NW, et al. Modeling normal and pathological ear cartilage in vitro using somatic stem cells in three-dimensional culture. *Front Cell Dev Biol* 2020;8: 666
74. Perrier-Groult E, Pérès E, Pasdeloup M, et al. Evaluation of the biocompatibility and stability of allogeneic tissue-engineered cartilage in humanized mice. *PLoS One* 2019;14:e0217183
75. Osiecka-Iwan A, Hyc A, Radomska-Lesniewska DM, et al. Antigenic and immunogenic properties of chondrocytes. Implications for chondrocyte therapeutic transplantation and pathogenesis of inflammatory and degenerative joint diseases. *Cent Eur J Immunol* 2018;43:209–219
76. Rahfoth B, Weisser J, Sternkopf F, et al. Transplantation of allograft chondrocytes embedded in agarose gel into cartilage defects of rabbits. *Osteoarthritis Cartilage* 1998;6:50–65
77. Langer F, Gross AE. Immunogenicity of allograft articular cartilage. *J Bone Joint Surg Am* 1974;56:297–304
78. Malejczyk J, Osiecka A, Hyc A, et al. Effect of immuno-suppression on rejection of cartilage formed by transplanted allogeneic rib chondrocytes in mice. *Clin Orthop Relat Res* 1991; 269:266–273
79. Kawabe N, Yoshinao M. The repair of full-thickness articular cartilage defects. Immune responses to reparative tissue formed by allogeneic growth plate chondrocyte implants. *Clin Orthop Relat Res* 1991;268:279–293
80. Mandl EW, van der Veen SW, Verhaar JAN, et al. Multiplication of human chondrocytes with low seeding densities accelerates cell yield without losing redifferentiation capacity. *Tissue Eng* 2004;10: 109–118
81. Van Osch GJVM, Mandl EW, Jahr H, et al. Considerations on the use of ear chondrocytes as donor chondrocytes for cartilage tissue engineering. *Biorheology* 2004;41:411–421
82. Bernstein JL, Cohen BP, Lin A, et al. Tissue engineering auricular cartilage using late passage human auricular chondrocytes. *Ann Plast Surg* 2018;80(suppl 4):S168–S173
83. Cohen BP, Hooper RC, Puetzer JL, et al. Long-term morphological and microarchitectural stability of tissue-engineered, patient-specific auricles in vivo. *Tissue Eng Part A* 2016;22:461–468

84. Domm C, Schünke M, Christesen K, et al. Redifferentiation of dedifferentiated bovine articular chondrocytes in alginate culture under low oxygen tension. *Osteoarthritis Cartilage* 2002;10:13–22
85. Ahmed N, Gan L, Nagy A, et al. Cartilage tissue formation using redifferentiated passaged chondrocytes in vitro. *Tissue Eng Part A* 2009;15:665–673
86. Ahmed N, Taylor DW, Wunder J, et al. Passaged human chondrocytes accumulate extracellular matrix when induced by bovine chondrocytes. *J Tissue Eng Regen Med* 2010;4:233–241
87. Tseng A, Pomerantseva I, Cronce MJ, et al. Extensively expanded auricular chondrocytes form neocartilage in vivo. *Cartilage* 2014; 5:241–251
88. Ko EC, Fujihara Y, Ogasawara T, et al. BMP-2 embedded atelocollagen scaffold for tissue-engineered cartilage cultured in the medium containing insulin and triiodothyronine—a new protocol for three-dimensional in vitro culture of human chondrocytes. *Tissue Eng Part C Methods* 2012;18:374–386
89. Arévalo-Silva CA, Cao Y, Vacanti M, et al. Influence of growth factors on tissue-engineered pediatric elastic cartilage. *Arch Otolaryngol Head Neck Surg* 2000;126:1234–1238
90. Baugé C, Boumédiene K. Use of adult stem cells for cartilage tissue engineering: current status and future developments. *Stem Cells Int* 2015;2015:438026
91. Pittenger MF. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;284:143–147
92. Dmitrieva RI, Minullina IR, Bilibina AA, et al. Bone marrow- and subcutaneous adipose tissue-derived mesenchymal stem cells: differences and similarities. *Cell Cycle* 2012;11:377–383
93. Fraser JK, Wulur I, Alfonso Z, et al. Fat tissue: an under-appreciated source of stem cells for biotechnology. *Trends Biotechnol* 2006;24:150–154
94. Gu Y, Kang N, Dong P, et al. Chondrocytes from congenital microtia possess an inferior capacity for in vivo cartilage regeneration to healthy ear chondrocytes. *J Tissue Eng Regen Med* 2018;12:e1737–e1746.
95. Tay AG, Farhadi J, Suetterlin R, et al. Cell yield, proliferation, and postexpansion differentiation capacity of human ear, nasal, and rib chondrocytes. *Tiss Eng* 2004;10:762–770
96. Childs RD, Nakao H, Isogai N, et al. An analytical study of neocartilage from microtia and otoplasty surgical remnants: a possible application for BMP7 in microtia development and regeneration. *PLoS One* 2020;15:e0234650
97. Schulz RM, Bader A. Cartilage tissue engineering and bioreactor systems for the cultivation and stimulation of chondrocytes. *Eur Biophys J* 2007;36:539–568
98. Krueger S, Achilles S, Zimmermann J, et al. Re-differentiation capacity of human chondrocytes in vitro following electrical stimulation with capacitively coupled fields. *J Clin Med* 2019;8: 1771.
99. Das RHJ, Jahr H, Verhaar JAN, et al. In vitro expansion affects the response of chondrocytes to mechanical stimulation. *Osteoarthritis Cartilage* 2008;16:385–391
100. Pound JC, Green DW, Chaudhuri JB, et al. Strategies to promote chondrogenesis and osteogenesis from human bone marrow cells and articular chondrocytes encapsulated in polysaccharide templates. *Tiss Eng* 2006;0:2789–99.
101. Hsu SH, Kuo CC, Whu SW, et al. The effect of ultrasound stimulation versus bioreactors on neocartilage formation in tissue engineering scaffolds seeded with human chondrocytes in vitro. *Biomol Eng* 2006;23:259–264
102. Grodzinsky AJ, Levenston ME, Jin M, et al. Cartilage tissue remodeling in response to mechanical forces. *Annu Rev Biomed Eng* 2000;2:691–713
103. Chung C, Erickson IE, Mauck RL, et al. Differential behavior of auricular and articular chondrocytes in hyaluronic acid hydrogels. *Tissue Eng Part A* 2008;14:1121–1131

