Generation of Ear Cartilage for Auricular Reconstruction

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Contents

Abstract

Auricular reconstruction is among the most challenging surgical procedures in plastic and reconstructive surgery because of the lack of an ideal auricle substitute that can guarantee a long-lasting outcome while involving minimal donor site morbidity. Tissue-engineered cartilage may provide an ideal autologous solution. After the first report on generation of human ear-shaped cartilage in a nude mouse model in 1997, cartilages with human ear shape have been engineered in vitro, in nude mice, and in immunocompetent animals using various cells and scaffolds. Recently, our group reported a pilot clinical trial of in vitro engineered human ear-shaped cartilage and its clinical translation for auricular reconstruction. This bench-to-bed process spanning over the past two decades can provide an in-

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depth understanding of the development of cartilage tissue engineering for auricular reconstruction. The current chapter will illustrate the research achievements and application challenges inherent in generating translational tissue-engineered cartilage for auricular reconstruction, particularly focusing on the global trends and new research directions of each associated building block or step, namely, seed cells, scaffolds, three-dimensional printing, in vitro microenvironment simulation, preclinical evaluation, and clinical translation.

1 Introduction

Auricular defects can be caused by congenital diseases such as microtia or by acquired defects due to burns, trauma, animal bites, tumor removal, or radiotherapy performed as a treatment for cancer. Microtia is a congenital malformation of the external ear, with a varied regional prevalence rate of 0.83–17.4 per 10,000 births worldwide (Paput et al. 2012; Bly et al. 2016). Compared to microtia, ear deformities caused by acquired defects, occurring in more than 1 per 500 people, may be more common (Jessop et al. 2016). The auricle is an important identifying feature of the human face, and hence, its deformity has a profound effect on both the level of self-confidence and continued psychological development in afflicted patients. The current cosmetic procedures for total auricular reconstruction mainly include the wearing of auricular prostheses or implantation of nonabsorbable auricular frame materials or an autologous rib cartilage framework (Bly et al. 2016; Jessop et al. 2016; Wiggenhauser et al. 2017; Zhou et al. 2018). Nonabsorbable frames composed of materials such as silastic or high-density polyethylene (Medpor) can generate an excellent ear shape without donor site morbidity, but they lack bioactivity and can lead to extrusion and infections. Alloplastic implants can be easily tailored with respect to shape and mechanical properties, but often lead to infection, incompatibility, and extrusion. Autologous rib cartilage transplantation is the current gold-standard treatment approach for external ear reconstruction owing to its good long-term durability as well as the potential to grow with the patient (Rosa et al. 2014). However, harvesting rib cartilage inevitably leads to donor site injury, and patients must have a sufficient supply of donor cartilage available to be eligible candidates for this procedure (Rosa et al. 2014). Moreover, replicating the complex three-dimensional (3D) ear structure is hard to achieve using surgeons' hand skills alone, which are highly dependent on their training background and clinical experience (Zhou et al. 2018).

Tissue-engineered auricles constitute a promising alternative to current ear reconstructive options. In 1997, the generation of engineered cartilage with a human auricular shape in a nude mouse model was reported by Cao et al. (1997). The aesthetic effect coupled with press coverage of this work gave people the impression that a tissue engineering-based solution for auricular reconstruction is just at the next corner, but only recently, our group led by Cao reported the first clinical translation of human ear-shaped cartilage (Zhou et al. 2018). The major efforts during this more than 20-year-long, bench-to-bed process lay in solving a number of problems,

including the lack of a proper cell source, the difficulty in generating ear-shaped cartilage with a predesigned 3D structure, the insufficient mechanical properties for shape maintenance, and the unfavorable host response to the engineered graft after its transplantation in vivo.

Developments in current cell biology, materials science, and advance manufacturing have contributed tremendously to solving the aforementioned problems and realizing the clinical translation of human ear-shaped cartilage. Although this tissue engineering-based approach still cannot shift the paradigm of conventional reconstructive methods, the lessons learned from the bench-to-bed process may provide deeper understanding and valuable experience to support further improvements in the quality of the engineered cartilage to better suit clinical situations by refining each building block of tissue engineering. The current chapter will outline the global trends and new research achievements of each building block or step in generating translational tissue-engineered cartilage for auricular reconstruction, including seed cell strategy, scaffold development, 3D printing, in vitro microenvironment simulation, and in vivo preclinical evaluations. Issues raised during clinical application that may direct future basic investigations to improve the quality of tissue-engineered cartilage are also discussed.

2 Traditional Approaches for Auricular Reconstruction

Many contemporary treatment options can be used for auricular reconstruction, including the wear of auricular prostheses, alloplastic framework implantation, and autologous cartilage transplantation. Alloplastic frameworks that are usually made from silastic or porous polyethylene (Medpor) are readily available with consistent levels of quality, are easy to work with, and require only a short operation time for placement. However, these materials lack bioactivity, and their mechanical properties are unmatched to those of the native auricular cartilage. Patients undergoing repair with Medpor implants experience complications at a rate ranging from 4% to 6.31% even when treated by the most experienced surgeons. Complications can include postoperative infections, skin perforations, framework fracture, compression ischemia, capsule fibrosis, and framework dislocations (Reighard et al. 2018). Meanwhile, the complication rate is even higher when using silastic implants (Berghaus 2007).

Autologous costal cartilage is currently the most biocompatible material available for total auricular reconstruction. The fundamental principles of current autologous cartilage transplantation strategies were first described in the English language by Gillies (1920). Tanzer revolutionized the surgical procedure by paring down the original six-stage method to encompass only three to four total surgeries, with subsequent technique modifications (Tanzer 1959; Reighard et al. 2018). Thereafter, Brent (1992, 2002), Park (Park et al. 1991; Park 2000), Nagata (1993), Firmin (1998), and Walton and Beahm (2002) contributed significantly to refining the surgical techniques used during this treatment (Olshinka et al. 2017). To date, this surgical approach has witnessed several notable advancements, including a

transition to a two- or even one-stage surgery, adoption of a minimally invasive approach to harvest rib cartilage, and imaging-assisted design of a more accurate patient-specific ear shape. However, although the procedure has advantages such as a high level of biocompatibility, long-term stability, immunocompatibility, and the potential for the implant to grow with the patient (Bichara et al. 2012), its conduct still requires special surgical experience, necessitates a considerable amount of operation time during which to shape the cartilage, and involves several reconstruction steps. In fact, it is among the most challenging procedures in plastic and reconstructive surgery, and only the most talented surgeons can consistently achieve satisfactory long-term postoperative outcomes (Jessop et al. 2016). Moreover, the age range of patients eligible to receive this surgery is also strictly restricted: the amount of costal cartilage can be insufficient in those who are younger than 6 years of age, whereas in patients who are too old, the costal cartilage may undergo calcification, and bending the cartilage without breaking it could become impossible (Kawanabe and Nagata 2006). The harvesting of the costal cartilage may also involve large donor site morbidity and induce significant pain, pneumonia, pneumothorax, atelectasis, and deformities of the rib cage and unattractive scars later (Ohara et al. 1997; Uppal et al. 2008). As a result, surgeons and researchers alike continue to search for alternative approaches by which to reconstruct the auricle.

3 Tissue Engineering Approach for Auricular Reconstruction

As a means to overcome the treatment obstacles of available surgical options, the reconstruction of human ear-shaped cartilage using tissue engineering techniques has attracted tremendous attention. Theoretically, a tissue-engineered auricle combines the merits of both an alloplastic framework and sculptured costal cartilage and possesses several advantages, including minimal donor site morbidity, accurate shape control, superb biocompatibility and bioactivity, short operation time (i.e., no need to scalpel the implant), and independence from the surgeon's level of experience. The first report on tissue-engineered human ear-shaped cartilage was published by Cao et al. (1997) who used bovine chondrocytes as seed cell, polyglycolic acid (PGA)/polylactic acid (PLA) as scaffold, and the immunodeficient nude mouse as an animal model (Cao et al. 1997). Although clinical translation took significant time thereafter to manifest, the aesthetic prominence of this work attracted massive media coverage, which led people to believe that the tissue-engineered auricle lay just around the next corner. In fact, however, it took about 20 years for Cao's group to realize the first clinical translation of human ear-shaped cartilage in five patients with microtia (Zhou et al. 2018), and still, this work retains some drawbacks such as long-term in vitro procedures and high costs, hindering its further and widespread clinical application and commercialization. To improve the quality of the engineered cartilage for auricular reconstruction, technologies relating to seed cell manipulation, scaffold preparation, in vitro cartilage regeneration, and in vivo preclinical evaluations need to be consistently refined. Similarly,

special surgical procedures for handling the engineered tissue also need to be developed.

3.1 Seed Cell Sources for Auricular Regeneration

The generation of a real-sized human ear-shaped cartilage requires the gathering of a significant amount of seed cells with a stable chondrogenic phenotype while simultaneously ensuring minimal donor site morbidity. Since the implantation site of the reconstructed auricle is a subcutaneous environment characterized by acute immune activity, seed cells of an autologous origin are needed to avoid immune rejection. Currently, the most intensively investigated seed cells for auricular reconstruction are auricular chondrocytes and mesenchymal stem cells (MSCs). Induced pluripotent stem cells (iPSCs) are under investigation but have yet to be used in translational studies.

3.1.1 Chondrocytes

Chondrocytes are the resident cells in the cartilage and therefore are the first choice as seed cells for cartilage tissue engineering. Chondrocytes can be divided into three types – elastic chondrocyte, hyaline chondrocyte, and fibrochondrocyte – according to the three types of cartilage where they are derived from, which are elastic cartilage, hyaline cartilage, and fibrocartilage, respectively. By adopting a developmental view, chondrocytes are derived from two embryological origins: the cranial neural crest (CNC) and the mesoderm (Taihi et al. 2019). More specifically, auricular (elastic cartilage) and nasal (hyaline cartilage) chondrocytes are derived from the CNC, whereas costal (fibrocartilage) and articular (hyaline cartilage) chondrocytes are derived from the mesoderm. Early studies on cartilage regeneration usually employed articular chondrocytes as seed cell candidates and largely found that they are nonproliferative, are prone to aging, and dedifferentiate after passage (Brittberg et al. 1994). Recently, accumulating evidence has suggested that CNC-derived chondrocytes (nasal or auricular chondrocytes) possess promising properties such as robust proliferation ability (Tay et al. 2004; Taihi et al. 2019). Moreover, these cells are more responsive to environmental cues than those derived from the mesoderm (Pelttari et al. 2014). After being treated with appropriate sources of stimulations such as basic fibroblast growth factor (bFGF) or low oxygen tension, the proliferation ability of those chondrocytes can be further enhanced, and a sufficient number of chondrocytes can be gained from a small biopsy sample collected from nasal or auricular cartilage (Zhou et al. 2018). In conditions with chondrogenic factors such as transforming growth factor-β1 (TGF-β1), those chondrocytes can regain their chondrogenic phenotype lost during extensive expansion and form stable cartilage in a nonchondrogenic subcutaneous environment (Zhou et al. 2018).

For auricular reconstruction, the application of auricular chondrocytes that can generate the elastic type of cartilage of the ear seems to be a reasonable choice. Particularly in patients with microtia, the microtic ear, which is usually discarded,

can instead be reused to provide seed cells for auricular regeneration (Kamil et al. 2004). In fact, all of the current clinical studies on auricular reconstruction based on cell therapy or tissue engineering report the use of auricular chondrocytes from the microtic ear (Yanaga et al. 2009, 2013; Zhou et al. 2018), although further investigations must be conducted to systematically compare microtic chondrocytes and normal healthy auricular chondrocytes. Recently, cartilage progenitor cells have been isolated from the perichondrium of the auricular cartilage, and these cells express stem cell markers such as CD44 and CD90 and possess even higher proliferation and chondrogenic capacities than those of chondrocytes isolated from auricular cartilage (Kobayashi et al. 2011; Kagimoto et al. 2016). This may allow an even smaller biopsy sample to be taken from the auricle perichondrium (without harming the ear cartilage), which can regenerate back after the biopsy, to yield sufficient chondrogenic cells to reconstruct the auricle.

3.1.2 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) were first reported by Friedenstein et al. (1968) and they are currently the most commonly used stem cells in human therapy and regenerative medicine. MSCs have gained considerable attention as seed cells for cartilage regeneration because of their ease of isolation (i.e., they are readily available), high proliferation potential, and great chondrogenic differentiation capacity (Huselstein et al. 2012). Moreover, MSCs have recently been found to possess immunomodulatory and anti-inflammatory properties, modulate lymphocyte cell function through the secretome, and exhibit several growth factors and cytokines, including TGF-β1, nitric oxide, interleukin-1 (IL)-1, and IL-10 (De Miguel et al. 2012). This immunomodulation is especially important when considering the acute immune reaction of the subcutaneous implantation site of the regenerated auricle.

MSCs can be derived from many sources, including bone marrow, adipose tissue, synovium, periosteum, umbilical cord blood, peripheral blood, skeletal muscle, and synovial fluid. Among the different types of MSCs, bone marrow-derived MSCs (BMSCs) have been considered to demonstrate the highest chondrogenic potential and are therefore the most frequently studied among seed cell candidates for auricular reconstruction. However, the cartilage engineered from BMSCs tends to undergo terminal ossification in the vascularized subcutaneous implantation site (Ichinose et al. 2005). In fact, ectopic ossification is a major problem restricting the application of MSCs to generate subcutaneous cartilage (De Bari et al. 2004). One possible reason for the phenotypic shift in MSC-derived cartilage is insufficient chondrogenic induction (Pelttari et al. 2006). To address this issue, a prolonged preinduction time, more robust chondrogenic growth factors, oxygen tension adjustments, and mechanical stimuli were applied to generate more sufficiently differentiated cartilage prior to implantation into the nonchondrogenic subcutaneous implantation site (Liu et al. 2008). However, it remains difficult to generate such a large piece of homogenous cartilage with a complex enough structure for auricular reconstruction through the chondrogenic induction of MSCs. Another important factor inducing ectopic ossification is the blood vessel invasion into the MSC-engineered graft (Liu et al. 2008). Evidence has revealed that BMSC-engineered

cartilage shows much higher expression levels of angiogenic factors, such as vascular endothelial growth factor (VEGF), and much lower expression levels of antiangiogenic factors such as chondromodulin-I (Chm-I), relative to its counterpart engineered from chondrocytes, inducing ingrowth of blood vessels into the MSC-engineered cartilage and triggering the ossification progress (Liu et al. 2008; Zhu et al. 2015). Approaches such as wrapping of a blood vessel-blocking membrane around the MSC-engineered cartilages or adopting scaffolds that can release antiangiogenic factors to engineer the cartilage have been studied to date (Li et al. 2017). However, these methods may also block nutrition transfer or have other negative effects on the seed cells, thus hampering the formation of homogenous cartilage with the required degree of quality.

3.1.3 Co-culture of Chondrocytes with MSCs

To address the issue of terminal ossification of MSCs, the co-culture of MSCs with mature chondrocytes has been investigated and found to be effective in generating phenotypically stable cartilage in the subcutaneous implantation site for the regenerated auricle (Zhang et al. 2014). Besides phenotype maintenance, co-culturing can effectively reduce the quantity demand for chondrocytes, which are limited in supply, while exert the merit of MSCs which are more readily available (Kang et al. 2013; Zhang et al. 2014). Kang et al. found that a co-culture arrangement of 30% chondrocytes and 70% BMSCs could generate ear-shaped cartilage in vitro (Kang et al. 2013). Zhang et al. reported the generation of stable subcutaneous human ear-shaped cartilage engineered through co-culturing of a 30% human microtia chondrocytes with 70% human BMSCs in a nude mouse model (Zhang et al. 2014). Moreover, co-culturing can generate specific types of cartilage: one study has detected elastic expression, the marker of the elastic cartilage of the auricle, in cartilage engineered by the co-culturing of BMSCs and auricular chondrocytes (Kang et al. 2012). An in vivo synergistic effect of MSCs and chondrocytes has also been reported by several groups, where a mixture of MSCs and chondrocytes proved to be more beneficial than chondrocytes or MSCs alone, and the researchers speculated that MSCs' immune regulation ability played a major role in the result (Kang et al. 2012; Ahmed et al. 2014).

There are currently several co-culture models being used – including mixed co-culture, separated co-culture, and both – to investigate the mechanism of co-culture capable of supporting stable chondrogenesis (Levorson et al. 2014; Morita et al. 2015). However, overall, the mechanism is still under debate. One possible mechanism is the chondrocytes' expression of the chondrogenic growth factors and antiangiogenic factors to support the chondrogenesis of BMSCs while suppressing hypertrophy and ossification. Some studies have observed a direct differentiation of chondrocyte-like cells in the cartilage lacuna of red fluorescent protein (RFP)-labeled MSCs (Zhang et al. 2014). Chondrogenic factors such as TGF-β1, bone morphogenetic protein-2 (BMP-2), and insulin growth factor-I (IGF-I) were found in the supernatant of chondrocyte-engineered constructs (Liu et al. 2010a). Other research, however, contends that the trophic factors secreted by MSCs support chondrocyte proliferation and cartilage formation (Wu et al. 2011, 2012; Wang

et al. 2013). These discrepancies in the literature may be explained by the variations in chondrocyte sources, scaffolds, and animal models used. A better understanding of these mechanisms is still needed to assess the efficacy of these co-culture systems, with particular attention paid to relative cell death in direct co-culture models. The state of the chondrocytes is also crucial to comprehend in this process (Graceffa et al. 2019). Aside from all of the intensive investigations, in clinical translation, the involvement of two types of cells together can induce complications and make standards and regulations difficult to establish. Therefore, translational studies remain more focused on identifying single-cell types for use as seed cells.

3.1.4 Pluripotent Stem Cells

Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and iPSCs, hold immense potential for regenerative purposes because of their properties of unlimited self-renewal and pluripotency. Further, they can represent a continuous sufficient source of seed cells of a committed lineage suitable for a larger-scale production for clinical applications (Cheng et al. 2014). ESCs were first isolated from the inner cell mass of mouse blastocyst-stage embryos and cultured as cell lines by Evans and Kaufman (1981) and Martin (1981), and the first established human ESC lines were derived from human blastocyst in 1998 (Thomson et al. 1998). In 2006, Takahashi et al. achieved a significant breakthrough by generating iPSCs through cell reprogramming (Takahashi and Yamanaka 2006), which brought into being an easily accessible source of pluripotent cells for autologous application while bypassing the ethical concerns related to the harvesting of human embryos, thus opening many gateways to progress in regenerative medicine research in cartilage tissue engineering.

The differentiation of PSCs into chondrocyte-like cells has been accomplished through several methods, including the formation of embryoid bodies, co-culturing, conditioned medium, and ESC- or iPSC-derived MSCs (Cheng et al. 2014; Gibson et al. 2017). Currently, chemically defined culture conditions have been established to generate a relatively pure chondrogenic population (without off-target differentiation or any residual PSCs) suitable for large-scale production (Cheng et al. 2014). However, one major concern that arises when using PSCs to engineer cartilage for auricular reconstruction is related to subcutaneous ossification. As mentioned above, MSCs with osteochondral-lineage plasticity are prone to undergoing terminal ossification and generally cannot maintain a stable cartilage phenotype in the subcutaneous implantation site. It is unknown at this time whether cartilage engineered from PSCs would also face the same problem (Castro-Vinuelas et al. 2018). Although studies have demonstrated that PSC-derived cartilage can be more phenotypically stable than MSC-derived cartilage (Castro-Vinuelas et al. 2018), the effects of these techniques in the setting of a subcutaneous implantation site remain relatively unexplored. Moreover, the use of iPSCs as seed cells to generate cartilage for clinical application still boasts safety issues and is currently under intensive investigation. Therefore, existing efforts are more focused on assessing more clinically relevant cell sources such as MSCs and chondrocytes for auricular reconstruction.

3.2 Scaffolds for Auricular Cartilage Regeneration

Scaffold as the structural building block of an engineered tissue provides a 3D template in which seed cells can grow and produce extracellular matrix (ECM). Hence, tissue engineering can control the shape of an engineered tissue mainly by controlling the shape of its scaffold. Moreover, some hydrogel or nanoscale scaffolds can provide a 3D microenvironment to help the chondrogenic seed cells maintain or regain the spherical chondrogenic characteristics (Benya and Shaffer 1982; Kimura et al. 1984; Bonaventure et al. 1994).

Since the auricle is a sophisticated 3D structure ultimately placed in a subcutaneous environment, scaffolds for engineering an auricle should meet certain requirements, including (1) high biocompatibility to support seed cell growth and cartilage formation in the subcutaneous implantation site characterized by acute immune reaction, (2) ease of accurate manufacturing into a complex 3D structure (even better if the scaffold can be created via 3D printing), and (3) strong mechanical properties for long-term maintenance of the predesigned 3D shape, particularly under skin tension after implantation.

Many types of materials have been used as scaffold to generate cartilage for auricular reconstruction, including synthetic materials, nature-derived materials, and combinations of different types of materials.

3.2.1 Synthetic Polymers as Scaffolds for Auricular Cartilage Engineering

Synthetic polymers such as PGA, PLA, poly(ε-caprolactone) (PCL), polyurethane (Chetty et al. 2008), and their copolymers in the format of sponges, fibrous mats, hydrogels, or electrospun fibrils have been used as scaffold materials for auricular cartilage engineering (Nayyer et al. 2012). Synthetic polymers can be massively produced with batch consistency, and their material properties can be custom-tailored via chemical and physical modifications. Further, they can easily be formed into the human ear shape through molding or other techniques and have relatively strong mechanical properties by which to maintain the predesigned shape. However, the biocompatibility of these synthetic polymer scaffolds is usually unfavorable for subcutaneous cartilage formation, and they lack the surface characteristics that favor cellular attachment and growth and are prone to inducing foreign-body reactions in the subcutaneous site of immunocompetent species, hence interfering with cartilage formation (Nayyer et al. 2012). Surface modifications of these scaffolds incorporating biosignaling molecules, cell-adhesion proteins, ECM components, growth factors, and anti-inflammatory factors have all been explored to optimize the biocompatibility of the said scaffolds. However, the majority of this work has focused on articular cartilage reconstruction, and studies testing these strategies in the setting of auricular reconstruction are lacking (Nayyer et al. 2012). In vitro precultivation, which facilitates sufficient degradation of scaffold before implantation, was demonstrated to be effective in reducing the foreign-body reaction induced by biodegradable synthetic scaffolds (Liu et al. 2016), but long-term in vitro culture is time-consuming and expensive and can

increase the risk of contamination. Therefore, attention has been paid to developing more biocompatible scaffolds to support subcutaneous cartilage regeneration, and nature-derived materials are generally considered to be more biocompatible than synthetic polymers.

3.2.2 Nature-Derived Materials as Scaffolds for Auricular Cartilage Engineering

The nature-derived materials that have been applied for auricular reconstruction include collagen, agarose, alginate, and decellularized cartilage ECM (Nayyer et al. 2012). These materials are usually found in the forms of sponge or hydrogel. The major advantage of using nature-derived materials is their superb biocompatibility by which to support cell attachment and low propensity to induce foreign-body reactions in the subcutaneous environment (Haisch 2010). For hydrogel scaffolds, the additional merits of the uniform encapsulation of seed cells and the ability to be formed into an accurate ear shape by injection molding exist (Faust et al. 2019). Cell-loaded hydrogels can also serve as a bio-ink for direct 3D printing of auricular-shaped constructs.

Among the nature-derived scaffold materials, calcium alginate and collagen have been successfully adopted to generate human ear-shaped cartilage in immunocompetent animals (Kamil et al. 2004). However, the mechanical properties of these scaffolds alone are too weak to maintain the sophisticated auricle shape, and stents or molds made from metal or other mechanically strong materials are usually applied to assist in shape maintenance. Although approaches such as surface modification and cross-linking can promote the mechanical properties of nature-derived scaffolds, the cross-linking media might increase the immunogenicity and cytotoxicity, and the mechanical properties of the reenforced scaffold are usually still too low for clinical application.

3.2.3 Combined Application of Materials as Scaffold for Auricular Cartilage Engineering

Since synthetic polymers generally lack biocompatibility and nature-derived materials generally lack mechanical properties, the combined application of different types of materials has been proposed to produce ear-shaped scaffolds with the desired biocompatibility and mechanical properties (Pedde et al. 2017). To maintain the reconstructed ear shape, the regenerated cartilage needs to possess a significantly high level of strength to resist tensions from the surrounding skin and scarring tissue. In this case, nondegradable inert metals such as gold or titanium have been used to assist mechanically weak materials to generate and maintain the human ear shape. Kamil et al. encapsulated mixtures of autologous chondrocyte and biodegradable polymers (i.e., calcium alginate, Pluronic F-127, and PGA) inside a human ear-shaped hollow gold mold prior to subcutaneous implantation into immunocompetent autologous hosts (swine or goat) and generated human ear-shaped cartilage with a normal anatomic definition (Kamil et al. 2004). An ear-shaped titanium stent was also used to assist mechanically inferior sheet materials (decellularized cartilage sheet or electrospun PCL sheet) to produce and maintain the ear shape (Gong et al.

2011; Xue et al. 2013). Pomerantseva et al. embedded a titanium wire into porous collagen to withstand mechanical forces and prevent shrinkage and distortion of the ear shape (Pomerantseva et al. 2016). However, these metal molds or stents cannot be regarded as intrinsic parts of the ear scaffolds given that they only play the role of physical support and do not have the ability to degrade upon the regeneration of cartilage tissue. Therefore, they either require additional surgery to be removed or present a high risk of extrusion as a metal foreign body even when they had been encased in the scaffold.

Recently, our group proposed the application of a 3D-printed PCL mesh as an inner core to replace the metal stent yet still endow the scaffold with the required mechanical strength and support for the ear shape (Zhou et al. 2018; Yin et al. 2020). PCL is a slowly degrading thermoplastic material with a low melting point (55–65 °C). Its mechanical strength can be finely tuned to approach that of a mature native cartilage by controlling the bar diameter of the 3D-printed PCL mesh. The PGA/PLA scaffold, demonstrated to support cell attachment and cartilage formation, can be easily wrapped around the PCL core through hot compression molding. It is worth noting that, owing to the low melting point of PCL, some portions of PGA fibers can fuse themselves into the PCL grids, making the PCL porous for cell infiltration after PGA/PLA degradation, which facilitates the replacement of the PCL core with the regenerated cartilage (Zhou et al. 2018). Besides PGA/PLA, nature-derived materials with better biocompatibility, such as decellularized cartilage matrix, collagen, or gelatin, can also be produced to bury the PCL core through freeze-drying and mold casting (Jia et al. 2020). These hybrid materials have shown positive results by inheriting suitable properties from both their natural precursors and PCL. Moreover, since PCL degrades slowly (the degradation time of PCL is mainly determined by its molecular weight, and the total degradation of PCL takes 3–5 years), the engineered cartilage may have sufficient time to mature and gain mechanical properties while gradually replacing the degrading inner core, which would avoid the trouble of removing the PCL stent and significantly lower the risk of stent extrusion. The combined application of different types of scaffolding materials such as PCL and hydrogel is also widely incorporated in the state-of-the-art 3D printing scenario, with PCL used to support the shape and hydrogel acting as a cell carrier.

3.3 3D Printing for Auricular Cartilage Engineering

3D printing is a process used to construct 3D objects by relying on the layer-by-layer deposition of materials onto a computer-controlled build platform (Pedde et al. 2017). In tissue engineering, 3D printing can facilitate the generation of tailor-made tissues with patient-specific geometry, through the combined use of medical imaging modalities, computer-aided design, and computer-aided manufacturing. Owing to its aesthetic impression and complex structure, human ear-shaped cartilage is frequently used as a research model to demonstrate the efficacy and superiority of 3D printing.

3D printing was originally introduced in auricular cartilage engineering to fabricate the scaffold into a patient-specific ear shape, with chondrogenic cells subsequently loaded into the scaffold for cartilage regeneration. Currently, 3D bioprinting has shown promise in the direct creation of tissue constructs, recapitulating the structural and cytoarchitectural complexities of native tissue through precise placement of cell-laden hydrogels in a layer-by-layer fashion (Kang et al. 2016). Many bioprinting techniques have been developed based on jetting, extrusion, laser-induced forward transfer (LIFT), and stereolithography (SLA) (Kang et al. 2016). The jetting method produces picoliter-scale drops with a printing resolution of 20–100 μm at high speeds (up to 10,000 droplets/s) and with a low cost, but the thickness of the printed constructs is limited because of the inadequate structural support garnered from the low-concentration, liquid-phase hydrogel being ejected (Pedde et al. 2017). Extrusion methods, which incorporate an air-pressure controller, a piston-assisted system, or a screw-assisted mechanism to continuously extrude biomaterials for layer-by-layer fabrication, can produce more stable 3D cell-laden structures using high-viscosity materials with higher cell densities (Malda et al. 2013; Wang et al. 2015), although the high printing resolution and speed achieved by involving high driving pressures and narrow nozzle diameters would create high nozzle shear forces that may reduce the cell viability (Murphy and Atala 2014; Kang et al. 2016). LIFT is a method originally developed to pattern metals and other inorganic materials onto a substrate (Pedde et al. 2017). The application of LIFT in tissue engineering has been reported to date in the fabrication of cellularized skin (Koch et al. 2012; Michael et al. 2013) and bone (Catros et al. 2011). Although LIFT is less commonly applied relative to other bioprinting systems because of its high cost, limited material versatility, low flow rates, and unwanted deposition of metallic residue, it has advantages, including high cell viabilities (exceeding 95%; Hopp et al. 2005), the precise printing of cells in relatively small constructs, and minimized clogging issues (Kang et al. 2016; Pedde et al. 2017). SLA is the highest resolution bioprinting approach currently available and relies on the irradiation of photopolymerizable macromer solutions using laser rastering or a dynamically projected light source to cross-link high-resolution patterns in the polymerization plane (Pedde et al. 2017; Melchels et al. 2010; Wang et al. 2015). Besides its high resolution, SLA is nozzle-free and can be low cost, but the widely used ultraviolet light inherent with this approach may reduce cell viability (although efforts have been made to replace the ultraviolet light with visible light), and the choices of photocurable materials with appropriate viscosities ($<$5 Pas) are limited (Melchels et al. 2010).

For the past 10 years, 3D printing has assisted in producing patient-specific ear-shaped scaffolds for subsequent chondrogenic cell seeding. Our group has used 3D printing-assisted approach to generate patient-specific ear-shaped scaffolds for the regeneration of human ear-shaped cartilage in vitro (Liu et al. 2010b), in nude mice (Zhang et al. 2014; Yin et al. 2020), and, recently, in a clinical setting (Zhou et al. 2018). Direct bioprinting of human ear-shaped cartilage has also been reported by a number of research groups using different scaffolds and bioprinting strategies (Otto et al. 2015; Kang et al. 2016), but their protocols have not yet been applied in

preclinical animal models or clinical scenarios. The translational value of these bioprinting techniques for auricular cartilage engineering needs to be further tested.

3.4 In Vitro Generation of Human Ear-Shaped Cartilage

One primary goal of cartilage tissue engineering is to provide surgeons with a piece of high-quality cartilage that is engineered in vitro and is thus readily available. As compared with the direct implantation of freshly seeded scaffolds, in vitro culturing may offer several advantages, including the following:

1. It allows sufficient ex vivo degradation of scaffolds that are otherwise prone to induce foreign-body reaction after implantation (Liu et al. 2016).
2. It allows stem cell preinduction to commit a stable chondrogenic lineage using exogenous growth factors (Liu et al. 2008).
3. It facilitates the generation of large grafts using a low density of seed cells (Liu et al. 2008).
4. The in vitro deposited ECM may enhance attachment of cells to the scaffold and may stabilize and protect the cells, improving seed cell retention and the maturation of engineered cartilage after transplantation (Ball et al. 2004; Deponti et al. 2012; Moretti et al. 2005).
5. It may simplify surgical handling, fixation, and postoperative treatment (Moretti et al. 2005).

Fortunately, the anural, aliphatic character of native cartilage makes it an ideal target tissue for in vitro cultivation. The following section will first introduce biochemical and mechanical stimuli – two of the most important factors influencing in vitro culture conditions for auricular cartilage regeneration. Other factors such as oxygen tension and in vitro culture duration will also be covered.

3.4.1 In Vitro Biochemical Stimuli

Biochemical stimuli in culture media can affect the quality of in vitro cultured cartilage by adjusting the status of seed cells and hence can affect in vivo cartilage formation after implantation (Okubo et al. 2019). To convert seed cells from the state of self-replication to that of chondrogenic differentiation or redifferentiation, culture media are usually divided into proliferation media and chondrogenic media, respectively, by applying different types of growth factors and other ingredients such as serum, hormones, vitamins, and other chemicals. Proliferation media are mainly applied to prime cells to reduce the cell doubling time, maintain the undifferentiated phenotype of stem cells, or recover the chondrogenic phenotype postexpansion, and chondrogenic media are mainly applied to increase the synthesis of cartilage-specific ECM, accelerate cartilage formation, and maintain a stable cartilage phenotype.

Growth factors play a pivotal role in regulating cell proliferation and chondrogenesis. In auricular cartilage engineering, the most widely used growth

factors include members in TGF-β superfamily (such as TGF-β1, TGF-β3, BMP-2, BMP-7), IGF-I, and bFGF (Okubo et al. 2019). TGF-β, BMPs, and IGFs are potent anabolic factors that can modulate cartilage metabolism and increase the deposition of cartilaginous ECM such as type II collagen and glycosaminoglycan (GAG). TGF-β can also maintain a chondrocyte phenotype and promote proliferation (Horton et al. 1989; Loeser and Shanker 2000; Grimaud et al. 2002). The chondroinductive actions of IGF-I were demonstrated to be equally potent to those of TGF-β1 for MSCs (Longobardi et al. 2006). IGF-I can stimulate proliferation, preserve chondrogenic potential, regulate cell apoptosis, and induce the expression of chondrocyte markers (Chiu et al. 2019; Shakibaei et al. 2006; Guenther et al. 1982; Loeser and Shanker 2000). Studies also suggest that IGF-I can improve the formation and localization of elastin (Rosa et al. 2014), which is a marker of elastic cartilage in the auricle (Chiu et al. 2019). However, IGF-I may promote the expression of collagen X, a marker of chondrocyte hypertrophy and mineralizing cartilages (Rosa et al. 2014). Meanwhile, bFGF mainly modulates cartilage metabolism (Loeser and Shanker 2000) and has been shown to elicit dose-dependent effects on chondrocyte mitotic activity but may also suppress proteoglycan synthesis (Sah et al. 1994). A synergic effect has been observed between these growth factors. TGF-β1 has been demonstrated to be able to synergistically catalyze the effect of IGF-I, and the impact of IGF-I on chondrogenesis was independent from that of TGF-β1 as indicated by the persistence of IGF-I's actions in MSCs lacking TGF-β1 signaling (Mauck et al. 2003; Longobardi et al. 2006). The combination of bFGF with insulin or IGF-I synergistically enhanced the proliferation of chondrocytes and MSCs (Munirah et al. 2010). Among these growth factors, bFGF and IGF-I are usually used in proliferation media, and TGF-β, BMPs, and IGF-I are usually used in chondrogenic media.

Besides growth factors, other medium supplements, including serum, insulin-transferrin-selenious acid premix (ITS premix), vitamin C, and dexamethasone, are usually relied upon to provide nutrition and support the growth factor function. Notably, conditions such as cell type, mechanical condition, and oxygen concentration (Jonitz et al. 2012) will influence the effects of the culture medium ingredients, and the diverse roles of ingredients may increase the level of difficulty when attempting to implement them in cartilage engineering (Trippel 1995; Faust et al. 2019).

3.4.2 Mechanical Stimuli in the In Vitro Culture Condition

Unlike articular cartilage, native auricular cartilage does not need to bear loads. However, the engineered auricular cartilage must possess a significant level of mechanical strength (more approximate to that of costal cartilage rather than that of native auricular cartilage) to effectively maintain the ear shape by resisting contractile forces stemming from the surrounding scarring soft tissue. As mentioned above, one direct way to significantly improve the immediate mechanical properties of the whole graft is by incorporating a mechanically strong scaffold. Another more bioinspired approach is the application of mechanical stimuli during in vitro culture to gradually improve the mechanical property of the regenerated cartilage tissue.

Various bioreactors, including a rotating-wall vessel, direct perfusion bioreactor, compression bioreactor, and spinner flask, have been introduced to provide static or dynamic mechanical stimuli in the forms of perfusion (laminar flow or turbulent flow), shear stress, compressive force, hydrostatic/hydrodynamic pressure, or their combinations (Concaro et al. 2009). These mechanical stimuli appear beneficial in promoting mass transfer within constructs (Moretti et al. 2005), activating growth factor signals (Yang and Barabino 2011), maintaining the spherical chondrocyte morphology, improving cell growth, regulating cell distribution, and permitting cartilage-specific ECM secretion (Graceffa et al. 2019). For the in vitro cultivation of a cartilaginous graft with a large volume and complex structure such as the auricle, these beneficial effects may translate to improved structural integrity (i.e., less void region in the central part) and enhanced mechanical properties (Vunjak-Novakovic et al. 1999; Faust et al. 2019).

However, for the current in vitro engineered cartilage, parameters such as GAG content, cell viability, and water content usually can be reached up to the level of native tissue, but the production and accumulation of some chondrocyte-specific ECM macromolecules – particularly collagen type II in cultured constructs – are insufficient when compared with native cartilage tissue, leading to the low mechanical properties of the in vitro engineered cartilage (Yan et al. 2009; Chen et al. 2014; Graceffa et al. 2019). Therefore, novel approaches need to be developed to allow more homogenous and mechanically robust cartilage regeneration in vitro.

3.4.3 Oxygen Tension

Physiologically speaking, articular cartilage is bathed in synovial fluid with low oxygen tension in joint capsule. Many studies have also reported the beneficial effect of a hypoxia environment (1–5%) on cartilage regeneration by enhancing cartilage-specific gene or protein expression, regulating apoptosis, and preventing terminal differentiation (Thoms et al. 2013; Browe et al. 2019), although the presence of hypoxia may also increase the expression of collagen type I (Lee et al. 2013). Nevertheless, the physiological subcutaneous environment for auricular cartilage is rich in nutrients and oxygen from the adjacent vascularized tissues (Moretti et al. 2005), and the effect of hypoxia is less emphasized in the context for auricular regeneration.

3.4.4 In Vitro Culture Duration

In vitro culture duration is an important factor that addresses the key question of how developed an engineered graft should be to support optimal cartilage repair or reconstruction (Moretti et al. 2005). However, no consensus has yet been reached on this matter. In a nude mouse study employing septal chondrocytes and PGA/PLA scaffold, Rotter et al. observed only minor differences in subcutaneous cartilage formation when the engineered constructs were precultured for either 1 day or 3 weeks (Rotter et al. 2002). In immunocompetent animal models (swine osteochondral model and rabbit or goat subcutaneous model) using autologous BMSCs or auricular chondrocytes as seed cells and PGA/PLA as scaffolds, our group found that the in vitro engineered cartilage displayed a time-dependent maturation process and prolonged in vitro precultivation (more than 2 weeks for

the rabbit subcutaneous model, more than 4 weeks for the swine osteochondral model, and more than 8 weeks for goat subcutaneous model), which could alleviate postimplantation inflammation and support stable cartilage formation (Luo et al. 2009; Liu et al. 2016; He et al. 2017). However, other groups (using nude mouse or autologous goat subcutaneous or osteochondral models, auricular or articular chondrocytes as seed cells, and fibrin, hyaluronic acid, or collagen as scaffolds) suggested that a short-term culture period (1–3 weeks) improved in vivo chondrogenesis whereas too long of a culture time led to worse results in spite of the existence of better maturation in vitro (Deponti et al. 2012; Miot et al. 2012; Bichara et al. 2014). This disparity could be explained by variables, including the different animal models (immunodeficient nude mouse vs. immunocompetent large animal), implantation sites (osteochondral vs. subcutaneous), scaffold types (synthetic scaffold vs. nature-derived scaffold), differentiation stages at the time of cell seeding, and cell-scaffold interactions (Moretti et al. 2005). Generally speaking, in an immunodeficient in vivo environment such as the nude mouse, or when using a biocompatible scaffold that would only induce mild foreign-body reactions in vivo, long-term precultivation is not necessary. Conversely, in immune-hostile environments such as the subcutaneous environment of immunocompetent species or when using a scaffold with suboptimal biocompatibility, prolonged in vitro precultivation could promote stable in vivo cartilage formation by allowing time for sufficient degradation of the inflammation inducing scaffolds. Moreover, if the seed cells appear in a suitable in vitro condition with good cell-scaffold interaction, prolonged in vitro culture would be more likely to promote in vivo cartilage development. Otherwise, too long of an in vitro culture could be harmful. Nevertheless, from a therapeutic perspective, long-term in vitro cultivation is labor-intensive and cost-prohibitive, may increase the risk of contamination, and may complicate the application process for approvals from regulatory agencies such as the US Food and Drug Administration. Therefore, future studies are encouraged to find ways to shorten the in vitro culture duration while generating mature in vitro cartilage for clinical application.

3.5 In Vivo and Preclinical Evaluations

Although in vitro studies reveal the potential of tissue-engineered auricles based on various cell sources, scaffolds, 3D printing, and biochemical and biomechanical stimuli, more in-depth knowledge regarding their in vivo fate must be gathered. For auricular reconstruction, a large number of in vivo studies continue to use the immunodeficient nude mouse as animal model (Nayyer et al. 2012). The nude mouse model plays an important role as an initial stage in evaluating the chondrogenic capacity of constructs in vivo and may provide a crucial link between in vitro investigations and complex and costly preclinical large animal studies (Moretti et al. 2005). Moreover, the nude mouse model supports the investigation of the in vivo fate of cartilaginous grafts engineered from human cells (Moretti et al. 2005). Following the first “ear-mouse” report in 1997 (Cao et al. 1997), the successful generation of human ear-shaped cartilage in the nude mouse model using different types of seed cells and scaffolds has been reported (Nayyer et al. 2012; Yin et al. 2020).

However, the subsequent efforts in a preclinical large animal model have been less successful because of the occurrence of immune attacks against the foreign scaffolds used to provide an auricular shape in the subcutaneous implantation site (Saim et al. 2000; Kamil et al. 2004; Shieh et al. 2004). To address this issue, hydrogel or nature-derived scaffolds with superb biocompatibility by which to support large animal subcutaneous cartilage formation were tested regarding the potential to generate human ear-shaped cartilage. Saim et al. injected Pluronic F-127 hydrogel loaded with chondrocytes into a skin-fold channel positioned in the shape of the auricle helix on the ventral surface of an autologous swine and generated helix-shaped cartilage (Saim et al. 2000). Chang et al. created chondrocyte-loaded alginate implants with specific shapes through an injection-molding process that formed mature cartilage after 6 months of subcutaneous implantation into autologous sheep (Chang et al. 2003). Kamil et al. delivered autologous chondrocytes inside an ear-shaped and ear-sized hollow gold mold in combination with calcium alginate or Pluronic F-127 and created a complete, anatomically refined auricle in a large animal model (Kamil et al. 2004). Pomerantseva et al. reported the growth of a stable, human ear-shaped cartilage in a preclinical sheep model using expanded chondrocytes and a titanium wire-supported collagen scaffold (Pomerantseva et al. 2016). However, the scaffolds adopted in these studies present weaker mechanical properties and may require the involvement of a metal mold or inner wire stent to support the ear shape, which will introduce additional complexity when removing the mold or will raise the risk of metal extrusion.

Recently, our group has developed a scaffold-free approach to generate a cartilage sheet in vitro. Without the involvement of the scaffold as a potential foreign body, these cartilage sheets exhibit superb biocompatibility, and we formed homogenous cartilage blocks of a large size (10 cm in diameter) and thickness (9 mm) by stacking several layers of sheets together and subcutaneously implanting them into an autologous goat. The regenerated cartilage block was then hand-carved into the auricle shape and reimplanted into the autologous goat for auricular reconstruction (Fig. 1, unpublished data). This study may address the issue of large donor site morbidity by using autologous auricular chondrocytes to generate the cartilage sheets since these cells are highly proliferative owing to their CNC origin (Tay et al. 2004; Taihi et al. 2019). To date, approaches for chondrogenic redifferentiation of postexpanded chondrocytes have been established by several groups (Yanaga et al. 2009; Pomerantseva et al. 2016; Zhou et al. 2018). However, the generation of the ear shape still relies upon the old-fashioned hand-carving skill.

3.6 Clinical Translation of Tissue-Engineered Cartilage for Auricular Reconstruction

Challenges restricting the bench-to-bed progress of tissue-engineered cartilage for auricular reconstruction are tremendous (Haisch 2010), and thus literature reports on this subject matter are very few and far between, especially when compared with those on cartilage engineering for orthopedic repair (Liu et al. 2017). Yanaga et al. described a two-stage approach where autologous chondrocytes (expanded in vitro)

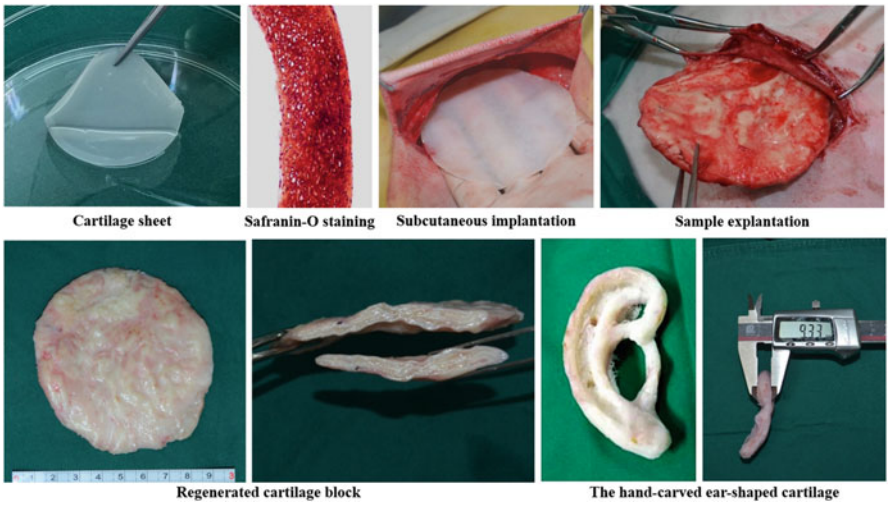

Fig. 1 Generation of human ear-shaped cartilage in an autologous goat model based on scaffold-free cartilage sheets. Cartilage sheets are generated by a scaffold-free approach. Five layers of the cartilage sheets are stacked and subcutaneously implanted into an autologous goat to generate a homogenous cartilage block of a large size (10 cm in diameter) and thickness (9 mm). The regenerated cartilage block was then hand-carved into the auricle shape

derived from the patient's microtic ear were injected into the lower abdomen of the patient to form cartilage blocks, which were then hand-carved into an ear framework to reconstruct the auricle for the patient (Yanaga et al. 2013). Recently, our group engineered patient-specific ear-shaped cartilage with proper mechanical strength in vitro based on expanded autologous microtia chondrocytes and a biodegradable PGA/PLA-PCL scaffold (Zhou et al. 2018). The regenerated cartilage was used for auricle reconstruction in five microtia patients using three different surgical approaches according to the specific condition of each patient. Postsurgical magnetic resonance imaging revealed the gradual degradation of the PCL inner core, and biopsies taken at 6 months postsurgery revealed mature elastic cartilage formation in all five patients. The achievement of this clinical translation can be attributed to the integration and innovation of several strategies, including using expanded autologous auricular chondrocytes as seed cell sources, establishing a chemically defined in vitro culture condition to alleviate the host's response toward the implanted graft, incorporating computer-aided design and manufacturing technique for patient-specific cartilage shape control, and adopting a PCL inner core for both immediate and long-term postoperative shape maintenance (Zhou et al. 2018).

Nevertheless, we still have a long way to go before tissue-engineered cartilage can be accepted as a clinically available treatment for auricular reconstruction. At present, the in vitro engineered ear graft (neocartilage) was still more delicate and fragile than the graft carved from fully developed rib cartilage, and the acute inflammatory trauma environment and the excessive handling during surgery, together with fibrogenesis of the surrounding soft tissue during wound healing,

may reduce the viability of the resident chondrocytes of the engineered ear graft, thus hindering the subsequent chondrogenesis and shape maintenance efforts after implantation. Therefore, surgical procedures need to be refined to work with the fragile tissue-engineered graft at this stage. To promote the widespread application of engineered ear grafts in the future, a backward bed-to-bench process to engineer a cartilaginous graft more proximate to the native cartilage is necessary (Martin et al. 2018).

4 Conclusions

Total reconstruction of the external ear is one of the most challenging procedures performed in the realm of plastic and reconstructive surgery because of the lack of an ideal graft presenting an accurate patient-specific ear shape, biocompatibility, and appropriate mechanical properties while inducing only minimal donor site morbidity (Nayyer et al. 2012). With the ongoing fast development in cell biology, materials science, engineering, and advanced manufacturing techniques such as 3D printing, tissue-engineered cartilage may constitute an alternative scheme for traditional ear reconstruction. The 1997 inaugural report coupled with the press coverage gave people the impression that a tissue engineering-based solution for auricular reconstruction is just around the next corner (Cao et al. 1997), but the reality is different. Obstacles restricting clinical translation still exist, including the lack of autologous seed cell sources with sufficient quantity and chondrogenic function and the lack of scaffold that is biocompatible to support subcutaneous cartilage regeneration and mechanically robust enough to resist skin tension. Recently, our group reported the in vitro generation of tissue-engineered cartilage and its clinical translation (Zhou et al. 2018), yet this tissue engineering solution still cannot replace the traditional approach. Even when we see the light of clinical translation, so to speak, new issues arise, such as how to better evaluate the seed cells and associated ex vivo expansion procedures to ensure long-term safety and function, how to generate a cartilage graft ex vivo with qualities and mechanical properties comparable to those of the native tissue, and how to refine the surgical procedures to better preserve both the viability and shape of the graft during surgical handling and after being implanted in the acute inflammatory trauma environment coupled with fibrogenesis of the surrounding soft tissue during wound healing. These issues support the need for a backward bed-to-bench process with the aim of further improving the quality of the engineered cartilage to better suit the clinical situation by refining each building block of tissue engineering (Martin et al. 2018). Endeavors in bioengineering and more in-depth collaborations between cell biologists, developmental scientists, materials experts, engineers, surgeons, and officers who conduct clinical and market entrance regulations are warranted to help tissue-engineered cartilage become a commercially viable and widespread alternative to autologous reconstruction.

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