FROM CELL TO SOCIETY

BIOENGINEERING AURICULAR CARTILAGE FOR EAR RECONSTRUCTION

IRIS OTTO

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FROM CELL TO SOCIETY:

Bioengineering auricular cartilage for ear reconstruction

VAN CEL NAAR MAATSCHAPPIJ:

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geboren op 14 juli 1988 te Utrecht

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INTRODUCTION

CHAPTER 1

INTRODUCTION, OBJECTIVES AND THESIS OUTLINE

The introduction is partly based on the following publication:
Zita M Jessop, Muhammed Javed, Iris A Otto, Emman J Combellack, Siân Morgan, Corstiaan C Breugem, Charles W Archer, Ilyas M Khan, William C Lineaweaver, Moshe Kon, Jos Malda, Iain S Whitaker.

Combining regenerative strategies to provide durable reconstructive options: auricular cartilage tissue engineering.

Stem Cell Research & Therapy (2016) 7: 19

INTRODUCTION

Ear deformities in a historical perspective

Throughout history, much value has been assigned to the appearance of the external ear, also known as auricle or pinna. Descriptions of an individual's character, including truthfulness, generosity, dullness and mental ability, were attributed to variations in size and shape of the ear [112]. Any mutilation or malformation of the ears was seen as an indication of unfitness or even of doom. Kings and priests with noticeable deformities were deemed unsuited to fulfill their role [112]. As early as several centuries BCE, congenital malformations of the auricle were observed, recorded and often accompanied with prophetic meanings. In Assyro-Babylonian culture, for example, auricular deformities came with superstitions about forthcoming disease, destruction and death [112]. In the Byzantine empire, the ruler was expected to satisfy the ideal of aesthetic perfection as a prerequisite to rule. Cutting off the ears of the emperor was therefore an effective way of removing him from the throne during an insurrection [188].

In her novel *Monkey Bridge*, Lan Cao wrote: "Ancient Chinese textbooks described the ear as a miracle organ. In the ear lay all the healing powers of the world." Western philosophers too, gave significant meaning to the ear. Voltaire pronounced "The ear is the avenue to the heart", which can be interpreted as the importance of listening as a means for human connection. Hippocrates laid the foundation for the belief that the ear is an organ of generation by postulating that auricular veins interrupted the flow of semen from the head to the genitalia. This belief can be observed in various (post-)medieval writings and theater plays [112]. Although contemporary medicine has refuted the ear as a reproductive organ, it may well serve as a source of regenerative capacities.

Microtia: a congenital auricular deformity

The auricle is a complex three-dimensional structure that features on both sides of the human cranium. It develops from the six hillocks of His at six weeks of gestation, which eventually fuse and form the intricate cartilage shape of the auricle. After birth, it continues to grow with the child, reaching adult size at around nine years of age [5]. The function of the auricle is to direct incoming soundwaves into the auditory canal.

Auricular deformities that are acquired in origin, caused by trauma, burns or cancer, occur in >1:500 of the population [159]. Less common are congenital anomalies: microtia has a prevalence rate of 1-17:10.000 births depending on geographical region [204]. Microtia is a congenital deformity where the auricular cartilage is underdeveloped, ranging from a mild structural anomaly to a complete absence of the auricle (anotia; Figure 1). More severe malformations are often accompanied by atresia (an abnormal narrowing) of the external auditory canal, which impedes conductive hearing [276]. The condition is most often seen in male patients. It is unilateral in 79-93%

of cases, with the right ear most commonly affected, but it can occur bilaterally [3, 138]. Microtia can exist as an isolated condition or together with a syndrome, such as hemifacial microsomia, Treacher Collins syndrome or Goldenhar syndrome [3, 56, 138, 203]. The etiology and pathogenesis of microtia are considered heterogenous and hypotheses for its development include single-gene mutations, neural crest cell disturbance, vascular disruption and altitude [203].

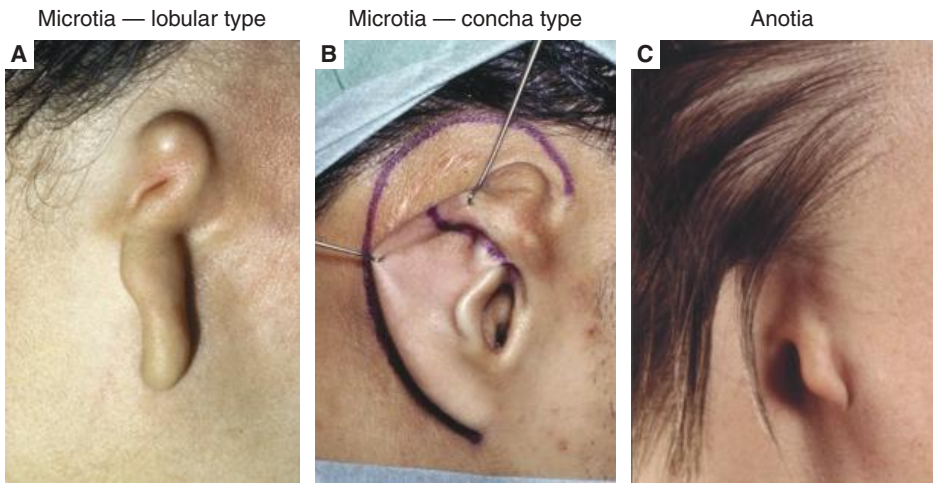

Figure 1. Classification of microtia according to Nagata. Microtia is a congenital structural anomaly of the human auricle. The classification by Nagata differentiates between lobular type microtia (A), (small or large) concha type microtia (B), and anotia (C) [343].

Reduced psychosocial well-being in patients with auricular malformations

Aesthetic perfection is still highly desirable in modern culture and deviations from the norm cause embarrassment and dissatisfaction [146]. Nowadays too, there are stigmas associated with auricular malformations [203] and teasing is a prominent aspect in the lives of both pediatric and adult microtia patients [146]. As such, abnormal appearance of the ears has a profound effect on self-confidence, quality of life and psychosocial development. Even minor disfigurements can cause psychological distress. Many patients with auricular deformities suffer from reduced self-esteem, increased anxiety and depressive mood states, as well as social withdrawal [146, 160, 162, 197, 287, 289]. Behavioral problems, including hostility and aggression, are also frequently reported in children with microtia [119, 146, 160, 197]. All these symptoms markedly affect patients' social lives and leisure activities [146, 160, 289]. Surgical correction of the malformation convincingly

leads to improvements in self-esteem, mood, social integration and general quality of life [119, 146, 162, 287, 321].

Current approaches for total auricular reconstruction and their limitations

Early approaches for reconstruction of the auricle involved transposed pedicled flaps or the implantation of silicone structures. However, these reconstructions were not very durable and proved prone to distortion and, in case of the latter technique, extrusion of the implant through the skin [17]. The benchmark work of Tanzer comprised a four-stage reconstruction technique using autologous costal cartilage [333-336]. This work was followed up by modifications by Brent [36-39], Park [273, 275], Nagata [235-239] and Firmin [103-105] for refinement of the surgical technique, improving the aesthetic result and decreasing post-operative complication rates [15].

Currently, a two-stage autologous auricular reconstruction, ideally performed in patients between eight to ten years of age, is the standard treatment worldwide. This approach uses the patient's own rib cartilage, harvested from the 6th to 9th ribs [207, 334], to supply donor tissue for the auricular framework. The first stage encompasses the sculpture of the framework to mimic the contours of the contralateral normal ear. After removal of cartilage remnants, wide mobilization of the skin and transposition of the lobule, the frame is placed in its proper position. In some patients the overlying skin needs to be expanded before inserting the auricular framework. In the second stage, the ear is mobilized and a cartilage block is placed posterior to the framework to accomplish projection from the head [103, 105, 236, 237].

Total auricular reconstruction is considered one of the most challenging operations in reconstructive surgery, largely due to the complex three-dimensional anatomy of the auricle [319]. The surgical technique requires significant artistic skill to shape the auricular framework. Matching the shape to the patient's contralateral normal ear occurs either by eye or with the help of image-acquisition technology [254, 277] or templates [58]. Consistently excellent results require a prolonged period of training to build up the necessary expertise.

The benefits of an autologous approach include immunocompatibility, high biocompatibility and long-term stability [159]. Nevertheless, the complex surgery is not without risk. A sufficient amount of cartilage needs to be harvested from the ribs in order to reconstruct a correct auricular framework. Morbidity at the donor site includes a permanent, sometimes hypertrophic scar [263, 350, 383]. Chest wall deformation [262, 350], clicking [350], pain [321, 350] and pneumothorax [341] are possible hazards. Sculpting the auricular framework requires experience and artistic talent, and does not always result in a satisfactory aesthetic outcome. In addition, stiffness due to calcification [39], exposure due to skin necrosis [104, 274], projection loss and distortion due to skin contraction

[104, 106], and cartilage resorption [20, 106, 365] are among the potential long-term complications associated with this type of surgery (Figure 2).

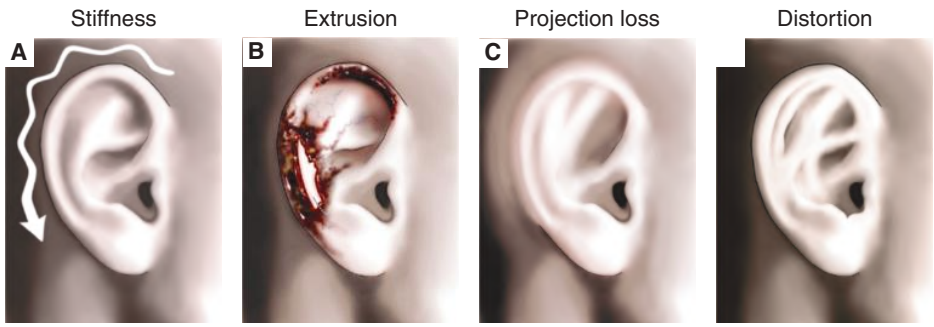

Figure 2. Long-term complications of autologous auricular reconstruction. Stiffness (A), extrusion (B), projection loss (C) and distortion (D) are among the risks associated with reconstructive surgery of the auricle.

Donor site morbidity can be avoided by using synthetic materials, of which porous polyethylene (brand name Medpor®; Stryker, USA) is getting increasingly popular among surgeons [15, 296-298, 332]. As no donor cartilage is required, this procedure can be performed at a much earlier age, even before children enter school [298]. Off-the-shelve shapes are available that require only minimal surgical adaptation to mimic the normal ear. The material is non-resorbable, non-toxic and provides a stable long-term shape [92]. However, the material is much more rigid than native auricular cartilage, causing an unnatural feel and risk of implant fracture [15, 92]. Extrusion due to infection or necrosis of the overlying soft tissue is also a serious possibility [53, 68]. Adequate coverage with a temporoparietal fascia flap is therefore required to minimize the risk of implant exposure [298]. The use of Medpor® has an even longer learning curve than autologous ear reconstruction and in unexperienced hands can lead to disastrous results. In that respect, costal cartilage is more forgiving in case of complications. In the autologous approach, exposure can often be treated with local flaps, while exposure of a Medpor® implant in most cases necessitates removal of the foreign material [195].

Another, yet much less applied alternative for auricular reconstruction is an osseointegrated implant with a synthetic prosthesis [15, 183]. The aesthetic results are considered excellent, yet its maintenance requires a long-term commitment from both patient and care team. As a new prosthesis is required every 2-5 years, it is a costly option [15]. Revision surgery, adverse skin reactions and implant failures were among the adverse effects in a long-term follow-up [183].

The regenerative approach to cartilage repair

Although the current reconstructive options can yield satisfactory aesthetic results for auricular repair, the limitations associated with each approach maintain an ongoing search for better alternatives. The field of regenerative medicine – specifically tissue engineering – has the potential to generate new tissue, using a combination of cells, biomaterials, and engineering methods [75]. In the 1990s, tissue engineering was established through the demonstration of neocartilage growth in a pre-shaped auricular structure in a rodent model [49]. Since then, there has been an incremental expansion of the applications of the technology for reconstructive surgery. Historically, tissue engineering has involved cell culture techniques, cell seeding of scaffolds to mimic extracellular matrix and growth of tissue in a bioreactor. These approaches have attempted to generate durable auricular cartilage replacements matching the functional and aesthetic properties of normal ears [41, 49, 136, 257, 295, 299, 315]. Although progress has been made and techniques have been refined, it is not yet possible to mimic the functional characteristics of native ears in terms of strength, flexibility and elasticity whilst maintaining the correct shape of the ear after insertion under the skin for prolonged periods of time [49, 168].

Biofabrication-based strategies for cartilage regeneration

Biofabrication is an emerging technology that, when combined with regenerative medicine approaches, has the potential to create custom-made tissue replacements for reconstructive surgery. It is based on three-dimensional (3D) printing technology, which creates objects from a digital model in a layer-by-layer fashion, offering full control over both external and internal architecture [81]. Biofabrication entails the creation of biological structures: it uses 3D printing in combination with living cells, bioactive molecules, biomaterials, micro-tissues or hybrid cell-material constructs for the automated generation of biologically functional products [132]. A computer-aided transfer process is used for patterning and assembling living and non-living materials with a prescribed three-dimensional organization. The resulting constructs can be subsequently matured into living structures that mimic the native tissue [219]. Biofabrication technology could offer a range of solutions for auricular reconstruction using the bioengineering approach.

OBJECTIVES AND THESIS OUTLINE

Recent advances in bioengineering and collaborations between stem cell biologists, engineers and clinicians have developed a landscape that provides the opportunity to engineer auricular cartilage constructs that resemble the human ear in shape, size and flexibility. Yet to this date, there is no clinically relevant treatment option using bioengineered cartilage for auricular repair. There are fundamental scientific questions that need to be addressed in order to overcome the current limitations of tissue-engineered constructs for long-term sustainability.

The central questions in this thesis are: *What are the challenges in bioengineering a neocartilage auricular implant, and how can these challenges be overcome?*

Addressing these challenges, the objectives are:

1. To identify an appropriate cell source for auricular cartilage tissue engineering
2. To fabricate an auricular scaffold for improved implant shape, stability and durability
3. To address ethical factors in biofabrication research and involve societal stakeholders for a more responsible research process

Chapter 2 reviews the main challenges in auricular cartilage tissue engineering: acquisition of sufficient numbers of cartilage-producing cells, creation and maintenance of the complex auricular shape, and provision of a supportive microenvironment. Biofabrication-based strategies are proposed to generate personalized shapes, combine materials in a spatially controlled environment, and improve mechanical stability of manufactured structures. These challenges form the basis for the further research in this thesis.

The first challenge is generating sufficient regenerative cells for cartilage tissue engineering strategies that can be used for reconstruction of the human auricle. Native chondrocytes require extensive expansion yet rapidly dedifferentiate [144, 288, 303, 307], whereas highly proliferative mesenchymal stromal cells display a preference for the endochondral ossification pathway [120, 229]. When applied for engineering of the human auricle, both cell types yield a neotissue of inadequate quality. In search of an appropriate autologous cell source that can yield sufficient cell numbers while maintaining cartilage-regenerating potential, **Chapter 3** investigates an auricular cartilage progenitor cell population (AuCPC) in the equine external ear. Promising results upon applying these cells in a 3D hydrogel culture system for cartilage engineering prompted the subsequent investigation of human auricular cartilage. **Chapter 4** identifies, characterizes and applies AuCPCs sourced from human adult and pediatric auricular cartilage, as well as the rudimentary microtia cartilage. This novel cell source offers many advantages for auricular cartilage tissue engineering.

The second challenge is to create the complex auricular shape and subsequently maintain it over time under the overlying skin. **Chapter 5** presents an imaging method to calculate the auricular surface area in a reliable manner. Due to the cartilage deficit, microtia patients exhibit insufficient skin to adequately cover a reconstructive implant. We determined a skin deficiency of >50% in microtia ears compared to the contralateral healthy auricle. The resulting contractive forces can cause pressing challenges for the underlying implant, underscoring the need for adequate support for maturing bioengineered cartilage. Mechanical reinforcement is believed to be required to help maintain the size and shape of the engineered auricular construct. In **Chapter 6**, the novel

human AuCPCs are printed together with a reinforcing scaffold in a customized auricular shape for a proof of concept of a printed hybrid ear. The potential clinical application of engineered auricular cartilage may well be a combination of various strategies.

An additional challenge is within the ethical domain: to predict the influence of these novel technologies on – and their interaction with – society. Scientific research in parallel with ethical analysis can anticipate impacts and stimulate a responsible research process. Responsible innovation encompasses the involvement of societal stakeholders in order to build effective bridges between research, clinic and society. **Chapter 7** outlines the ethical factors associated with biofabrication that require attention within these spheres, including the use of cells and animals, designing appropriate clinical trials, and expectations and concerns of society. Without involvement of societal stakeholders, research processes and outcomes may be misaligned with societal values and needs. Therefore, taking the step towards improving stakeholder involvement, **Chapter 8** evaluates the attitudes of parents of children with microtia towards the novel technologies being researched for future auricular cartilage repair.

These studies are summarized in **Chapter 9**, where the major findings are placed in context and future perspectives are discussed.

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CHAPTER 2

AURICULAR RECONSTRUCTION USING BIOFABRICATION-BASED TISSUE ENGINEERING STRATEGIES

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Biofabrication (2015) 7: 032001

ABSTRACT

Auricular malformations, which impose a significant social and psychological burden, are currently treated using ear prostheses, synthetic implants or autologous implants derived from rib cartilage. Advances in the field of regenerative medicine and biofabrication provide the possibility to engineer functional cartilage with intricate architectures and complex shapes using patient-derived or donor cells. However, the development of a successful auricular cartilage implant still faces a number of challenges. These challenges include the generation of a functional biochemical matrix, the fabrication of a customized anatomical shape, and maintenance of that shape. Biofabrication technologies may have the potential to overcome these challenges due to their ability to reproducibly deposit multiple materials in complex geometries in a highly controllable manner. This topical review summarizes this potential of biofabrication technologies for the generation of implants for auricular reconstruction. In particular, it aims to discuss how biofabrication technologies, although still in pre-clinical phase, could overcome the challenges of generating and maintaining the desired auricular shapes. Finally, remaining bottlenecks and future directions are discussed.

INTRODUCTION

Auricular malformations, as a result of congenital anomalies, trauma, burns or cancer, impose a significant social and psychological burden on the patient [319]. Improved psychosocial aspects have been documented after auricular reconstruction [321, 328]. Current treatment options include ear prostheses, synthetic implants and auricular reconstruction using skin flaps or autologous rib cartilage. The very first mention of ear reconstruction already dates back to the 6th century BC: the Sushruta Samhita, a Sanskrit text on surgical techniques, describes a cheek flap for earlobe repair [24]. In the 16th and 19th century, various other skin flaps have been used for the partial reconstruction of (traumatic) ear deformities [83, 331]. In the early 20th century, techniques have been introduced for complete ear reconstruction. However, these approaches, which used diced and molded rib cartilage, were challenged by progressive resorption [69, 284, 322, 379]. In 1937, Gillies even described the repair of more than 30 congenitally malformed external ears (microtia) using ear cartilage from the patient's mother. This approach did, however, not overcome the resorption issues [126]. A major breakthrough in the field of auricular reconstruction came in the form of a carved solid block of autogenous rib cartilage and was introduced by Tanzer in the late 1950s [333]. Modifications of this technique are still regarded the gold standard for auricular reconstruction in patients with microtia [295, 315, 373].

Auricular reconstruction with autologous costal cartilage is, nevertheless, considered an especially challenging procedure in plastic surgery because of the complex three-dimensional (3D) shape of the auricle [17, 25, 32, 56, 65, 199, 315]. Carving the auricular framework based on the contralateral healthy ear requires significant surgical skill. Differences in surgeon experience, the technique used and tissue handling, together with unpredictable scar tissue formation, account for marked variability in aesthetic outcome. When creating an auricular implant, the surgeon should emphasize the eminences and depressions of the human auricle (Figure 1), as the overlying skin, which is usually thicker than the skin on the normal ear, will reduce such details [17]. After reconstruction surgery, it also remains a substantial challenge to maintain the shape of the implant. Costal cartilage, harboring no elastic fibers, lacks the flexibility of a normal ear and can, therefore, appear rigid. In addition, harvesting sufficient amounts of costal cartilage for the hand-carved autogenous implant involves surgery with significant operating time and results in donor site morbidity [65, 154, 242, 295, 315, 373].

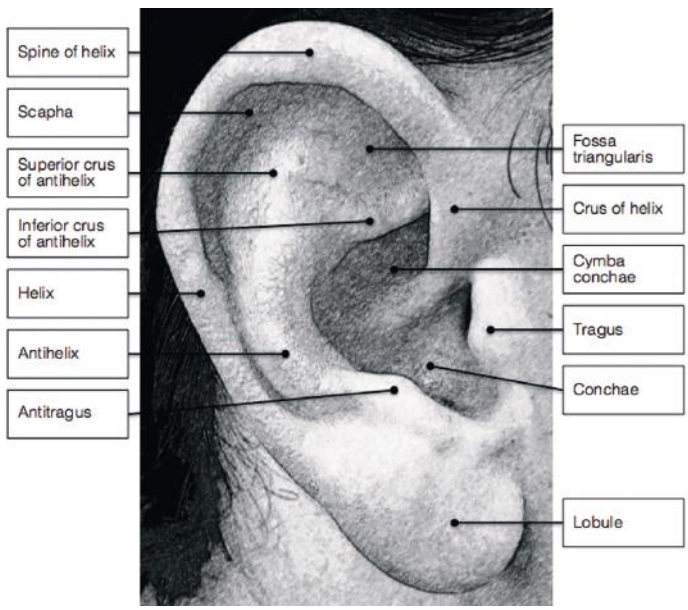

Figure 1. Anatomy of the human auricle. The unique three-dimensional shape of the human auricle emphasizes the eminences and depressions. Key elements are the helix, antihelix, concha bowl, tragus and antitragus.

In order to address the increased operating time, donor site morbidity, and intersurgeon shape variability, efforts have been made towards creating prefabricated synthetic auricular implants, including silicone ear frameworks and implants based on nylon and teflon [71]. Nonetheless, it appeared that these non-degradable synthetic implants were at high risk of extrusion secondary to infection or trauma and were, therefore, deemed unsuitable for reconstruction of the auricle [17, 206, 242, 315, 328, 337, 342] Medpor®, a porous high-density polyethylene implant, also evoked concerns of implant exposure, but has regained interest in the past few years when combined with temporoparietal fascial flaps and skin grafts [178, 328]. However, a recent international survey among plastic surgeons showed that the great majority prefers the use of autologous cartilage frameworks over such synthetic implants [40]. Nevertheless, the disadvantages of the current treatment modalities call for a further exploration of alternatives.

Advances in the field of regenerative medicine provide the possibility to engineer functional cartilage using patient-derived or donor cells, overcoming potential rejection of the neo-tissue [80, 232]. Such durable cartilage structures can also be generated from auricular cartilage cells and the resulting constructs could be used as auricular implant replacements [25]. Moreover, the convergence of technologies leading to the rapid advancements within the field of biofabrication now allows for the creation of cell-laden implants with intricate architectures and complex shapes [364, 391]. This approach would avoid patient donor site morbidity and other limitations associated with harvesting costal cartilage and manually sculpting an ear-shaped framework [25, 328]. Based on 3D imaging, implants can be custom-designed to closely match the contralateral ear, resulting in both improved aesthetic and functional outcomes [391]. This review summarizes the potential of biofabrication technologies for the generation of implants for auricular reconstruction. In particular, it aims to discuss how biofabrication technologies, although still in pre-clinical phase, could overcome the challenges of generating and maintaining the desired auricular shapes. Finally, remaining bottlenecks and future directions are discussed.

CHALLENGES IN THE GENERATION OF REGENERATIVE AURICULAR IMPLANTS

Engineering a pre-formed auricle that contains living cells dates back to the 1940s, where diced cartilage grafts and external molds in predetermined ear shapes were used for *in vivo* tissue repair [285]. In the 1970s, after a series of – albeit unsuccessful – experiments, the belief arose that appropriate scaffolds could coax cells into generating new tissue [129, 352]. A decade later, the use of synthetic biocompatible, biodegradable polymers as a temporary support structure was suggested [48, 186] and the feasibility of generating 3D cartilage constructs was demonstrated by seeding isolated chondrocytes on a fibrous polyglycolic acid (PGA) scaffold [48] This approach resulted in significant cellular growth and matrix production *in vitro*. Moreover, extended

incubation *in vivo* demonstrated histological resemblance to cartilage and maintenance of the 3D shape of the construct [109]. Additional experiments also confirmed that small cell-seeded polymer constructs implanted in nude mice progressively degraded and gradually were – almost entirely – replaced by neo-cartilage. In contrast, control groups with polymer alone or cells alone did not demonstrate new cartilage tissue formation [186]. Although neocartilage was produced within these small constructs, growing tissue engineered auricular cartilage in a particular complex 3D shape, such as the human auricle, remained a significant challenge [351]. Nearing the end of the century, a major breakthrough was achieved by implanting an engineered ear on the dorsum of nude mice [49]. This new approach involved a mesh of PGA immersed in polylactic acid, shaped in the form of a human ear, and subsequently seeded with articular chondrocytes. After 12 weeks *in vivo*, implants that were stented externally looked nearly identical to the initial implant. Removal of the skin revealed that a neocartilage framework had actually formed, which was responsible for the – at least temporary – maintenance of shape after removal of the external stent. Implants that were not initially stented externally faced a reduction in size and shape deformation [49].

These early experiments have paved the way for growing interest in the use of tissue engineering technologies for the generation of viable auricular implants. The ideal engineered implant should durably match the shape of the contralateral auricle, incorporating autologous chondrocytes or stem cells that have matured into native-like neocartilage tissue, which is strong enough to withstand the contractive forces of the skin and to enable the natural elastic bending of the auricle [25, 54]. With time, the scaffold material should slowly degrade while new cartilaginous matrix replaces it, maintaining its original shape [25, 209, 295]. Next, an auricular implant could even incorporate fatty tissue, perichondrium, or even the covering skin besides the cartilage framework [190]. Taken together, the major challenges faced in the generation of a regenerative auricular implant include the provision a proper environment for tissue growth, remodeling and maturation, the replication and maintenance of the auricular shape, and the generation of constructs that consist of multiple (pre-)tissues.

Microenvironment

It has been suggested that between 100 and 150 million cartilage cells are required to reconstruct an adult ear [25] and this entire mass of developing cartilage is primarily dependent on diffusion for the supply of oxygen and nutrients. In the native auricle, the cartilage lacks a vascular network and the perichondrium, a thin connective tissue layer surrounding auricular cartilage, is essential in facilitating blood supply to the cartilage surface [32]. Cultured cartilage however, being devoid of a perichondrial layer, completely lacks this vascular supply at the surface in the crucial early stages of development *in vivo*. In particular within larger cartilage constructs, such as the human auricle, this inevitably leads to profound problems with cell viability and proliferation [32], resulting in inhomogeneous tissue formation [196] and central necrosis [208].

Besides an adequate supply of nutrients, a stimulatory environment is essential for cell growth, proper differentiation and matrix production. Tissue engineering traditionally involves a mixture of cells, supporting scaffolds, and bioactive cues, *e.g.* growth factors, and the ideal composition of this mixture potentially allows for optimal tissue development [187, 324]. Chondrocytes typically thrive best in a soft hydrogel, a highly aqueous cell carrier that allows unimpeded nutrient diffusion and provides a homogenous microenvironment harboring stimulatory components for cellular migration, proliferation and differentiation. Temporarily simulating the natural extracellular matrix of the tissue, hydrogels serve as a guiding support structure for the deposition of new matrix [97, 209]. Just as in naturally developing tissue, cells in engineered constructs – both chondrocytes and stem cells – require guidance of bioactive cues to differentiate towards the (auricular) chondrogenic lineage. Insulin growth factor (IGF), the fibroblast growth factor (FGF) family and the transforming growth factor beta (TGF-β) family appear to be crucial in the development of cartilage tissue [172].

Creating the shape

The human auricle is a complex 3D shape that includes eminences and depressions formed by the outer helical rim, Y-shaped antihelix, concha bowl, tragus and antitragus (Figure 1). As for reconstructive surgery using costal cartilage, accurately mimicking the shape of the auricle under the skin is also a major challenge for auricular reconstruction using a tissue engineered implant. Several approaches have been adopted for the generation of engineered cartilage in the shape of the human ear. Many studies have applied hand sculpted and impression molds for the creation of the complex 3D shape of the external ear (Figure 2) [41, 154, 168, 315, 373]. Molds were, for example, injected with a hydrogel scaffold [168], or polymer sheets seeded with a cell suspension were placed in the mold [41, 315] or on a positive cast [154]. Although the initial constructs resembled the shape of the human ear, special attention must be drawn to highlighting the existing eminences and depressions of the auricular framework in order to create a pleasing aesthetic outcome.

Maintaining the shape

Although neocartilage production has been achieved within various scaffold materials and initial satisfactory aesthetic results have also been reported, the majority of studies on bioengineered auricular implants *in vivo* have faced degradation and deformation issues [41, 315], exemplifying the need for some form of support during the maturation of the new tissue [49, 246, 373]. The poor mechanical strength of the construct is partly due to the limited physical properties of the highly aqueous hydrogels. Despite the increased mechanical properties, stiff hydrogels are undesirable as they hinder the cellular processes required for tissue development [98, 209, 314]. Consequently, it is not surprising that internal support structures, including wire frameworks or polymer scaffolds, have yielded better results with regards to shape maintenance of newly formed cartilage (Figure 3) [25, 54, 136, 167, 201, 389].

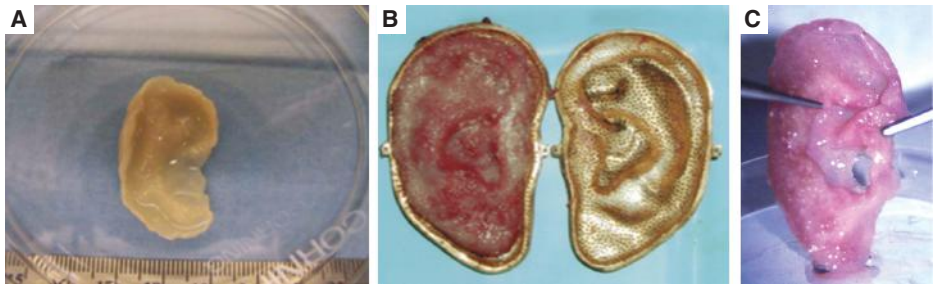

Figure 2. Examples of tissue engineered ear shapes using molds. A PGA/PLLA mesh was shaped using a negative mold and seeded with chondrocytes (A). Reprinted with permission from Shieh *et al.* (2004) [315]. Reproduced with permission of Elsevier. A gold negative mold was filled with chondrocytes mixed with various biodegradable polymers (B). Reprinted with permission from Kamil *et al.* (2004) [168]. Reproduced with permission of John Wiley and Sons. A silicone mold was filled with a PLLA/PGLA polymer scaffold and seeded with chondrocytes (C). Reprinted with permission from Haisch *et al.* (2002) [136]. Reproduced with permission of Springer.

An additional factor that may contribute to the degradation and deformation of engineered constructs is the hampered tissue maturation as a result of limited nutrient supply. In addition, immature and dysmorphic cartilage exhibits significantly less strength than healthy mature cartilage, and is therefore likely to face degradation *in vivo*. This may be of specific importance for larger constructs, *e.g.* for the replacement of an entire auricle, as these are likely to suffer from central cell death and limited proliferation due to nutrient limitation [32].

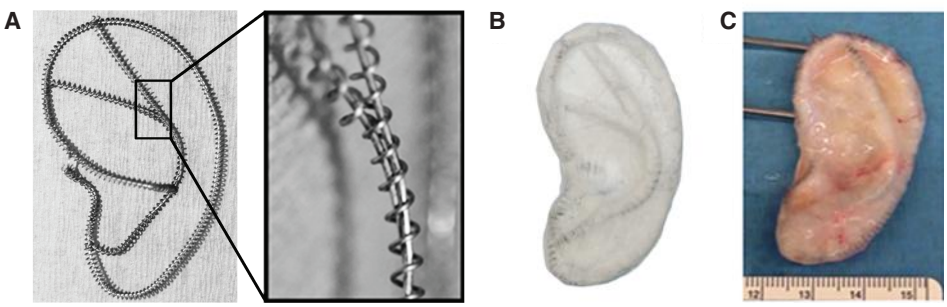

Figure 3. Auricular structure with internal mechanical support. An ear-shaped metal wire framework (A) combined with collagen (B) as an internal support structure to maintain dimensions (C). Reprinted with permission from Cervantes *et al.* (2013) [54]. Reproduced with permission of the Royal Society.

Hydrogel-based constructs will exhibit considerably less stiffness than native cartilage tissue [209]. Pre-culture, or 'maturation' of constructs before implantation, will improve strength due to

matrix deposition. Nevertheless, *in vitro* engineered cartilage constructs do still not have sufficient strength to withstand the contractive forces of the skin [17, 376, 389]. To date, studies incorporating internal support structures have yielded better outcomes with regards to maintaining dimensions and contours [54, 136, 167, 201, 376, 389, 391].

BIOFABRICATION-BASED STRATEGIES FOR AURICULAR RECONSTRUCTION

The engineering of auricular cartilage constructs thus faces many challenges, including functional biochemical composition, satisfactory anatomy, the creation of a customized shape, and especially the maintenance of that shape. Biofabrication technologies may have the potential to overcome these challenges due to their ability to deposit multiple materials in complex geometries in a highly controllable manner.

Microenvironment

A key issue in tissue engineering is providing the right local cellular environment that promotes cell growth, proper differentiation, and matrix production. Biofabrication technologies can deliver a hybrid construct of the various materials that are required to provide such an environment with high spatial resolution [209]. Hydrogels can function as a building block, as well as a carrier for the cells [219]. These 'bio-inks' provide a natural aqueous environment for the cells and have the advantage that they can be processed into a particular shape through biofabrication (Figure 4).

An additional exciting option is using a soluble and printable form of decellularized extracellular matrix (dECM) to create a favorable microenvironment for the encapsulated cells [281, 362]. As dECM contains all components of a natural cell environment, it has the potential to greatly enhance cell adhesion, proliferation, organization and maturation [18]. Tissue-specific dECM facilitates specific tissue formation and remodeling, and directs stem cell differentiation and commitment to the determined cell lineage [281].

Biofabrication technologies can further contribute to the appropriate complexity of the microenvironment for cell growth and differentiation through the delivery of spatially distributed gradients of biochemical cues. For example, various growth factors, including fibroblast growth factor-2 (FGF2), TGF-β and IGF, could be incorporated and their spatiotemporal release profiles could be tailored towards optimal tissue synthesis and maturation [293].

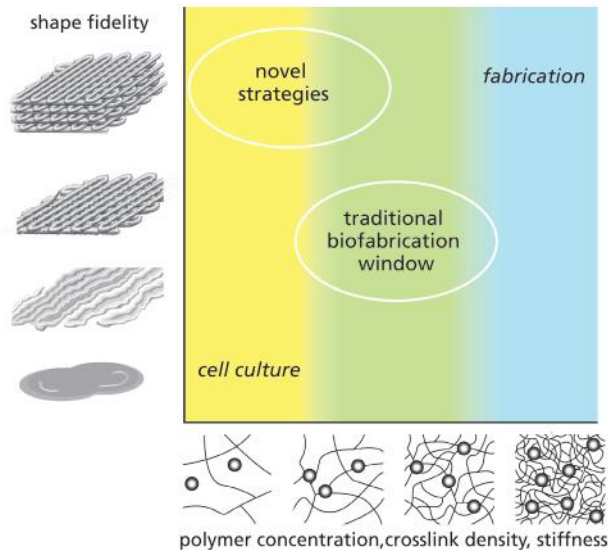

Figure 4. The biofabrication window. High printing accuracy is typically achieved with stiff hydrogels that contain high polymer concentrations and/or cross link densities (fabrication window). Cellular proliferation, migration and differentiation is generally enhanced in soft hydrogels (cell culture window), which are less suitable for fabrication of stable shapes stiffness is sufficient mechanical stability and optimal cell conditions. The traditional biofabrication window compromises on both physical and biological properties, yet novel strategies should seek materials and techniques that can attain high shape fidelity of the constructs while maintaining the ideal cellular environment. Reprinted with permission from Malda *et al.* (2013) [209]. Reproduced with permission of John Wiley and Sons.

Creating the shape

As discussed earlier, accurately mimicking the complex auricular shape is one of the challenges in creating a suitable auricular implant. The medical field already makes use of advanced imaging techniques, such as computed tomography (CT), magnetic resonance imaging (MRI), surface scanning and 3D photography, that are available to aid biofabrication processes through computer-aided design (CAD) and manufacturing (CAM). CAD/CAM technologies can precisely determine the original auricle shape and transform the 3D image data into a manufacturing output file for biofabrication [14, 122, 241]. Image-guided design and fabrication has already been used to create meniscus [14] and ear molds for hydrogel-based constructs with fine details [295]. Although such molds allow for gentle shaping of a single cell-seeded material, they do not allow the control of internal material or cellular variations [55]. Recently, patient-specific porous scaffolds of the auricle and nasal tip have been created with CAD/CAM technologies, using CT imaging and a laser-based 3D printing process. These porous scaffolds were subsequently filled with a cell-seeded hydrogel using a custom-designed mold [391].

Direct deposition of cell-containing hydrogels does allow for the generation of constructs with highly controllable and potentially porous complex configurations that closely resemble native tissue architectures [55, 81, 311]. With biofabrication technologies, a high patterning resolution, as well as precise spatial organization of the cellular environment can be achieved using the digital blueprint of a tissue. Different extracellular matrix components, cell types and bioactive molecules, as well as solid biodegradable materials and hydrogels, can be co-deposited into a specific heterogeneous configuration [219]. The feasibility of such an approach has been demonstrated through the fabrication of a construct consisting of an auricular cartilage framework and fatty tissue earlobe, using co-deposition of two different cell-laden hydrogels within an ear-shaped PCL framework (Figure 5) [190].

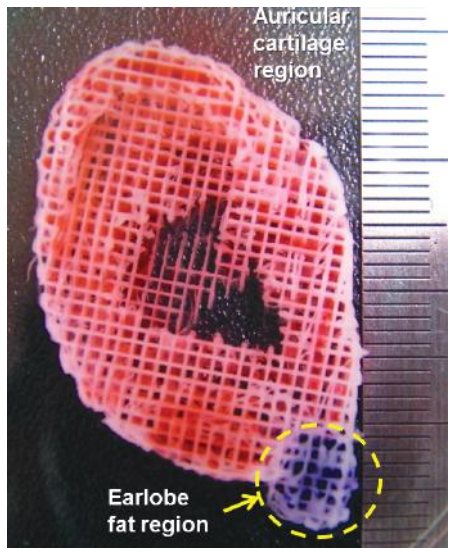

Figure 5. Auricular construct composed of multiple tissue types. A composite tissue was attained by the co-deposition of multiple hydrogels within the auricular three-dimensional (3D) shape. Reprinted with permission from Lee *et al.* (2014) [190]. Reproduced with permission of IOP Publishing.

The complexity of the shape of the auricle does pose limitations on the building of the implant. One limitation of additive manufacturing techniques is an increase in horizontal cross-sectional area with height, which is the case for the auricle from every angle. The resulting overhangs complicate the printing process. In order to create such a shape without collapsing, either temporary support structures have to be generated during the fabrication process or – alternatively – the construct has to be divided into smaller modules. Alginate [364], Pluronic F127 [181] and poly(ethylene) glycol (PEG) [190] are examples of sacrificial support materials that can be applied in biofabrication processes, without having notable detrimental effects on cell viability. In the case of smaller

modules, the design of each part should exhibit decreasing horizontal cross-sectional area, so that no sacrificial support layers are required (Figure 6). The parts can later be merged to generate a complete implant.

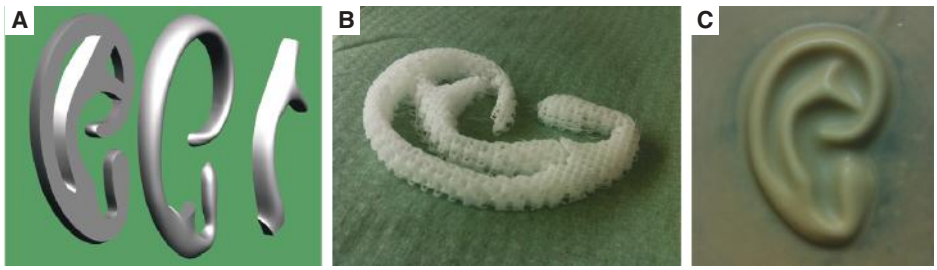

Figure 6. Modular approach to engineering the human auricle. In this approach, the open framework auricular model is divided into separate modules (A). Each module exhibits decreasing cross-sectional diameter and allowance of adequate oxygen gradient. Separate modules printed in PCL can be assembled to form complete construct (B). The assembled modular framework displays a satisfactory aesthetic appearance under rubber 'skin' (C).

Image-guided design can aid in developing an auricular implant closely resembling the anatomy of the patient. Recently, a parametric model that is fully customizable to the wishes of the patient and requirements of the surgeon was developed for the conversion of image data into a patient-specific manufacturing output file [33]. Thus, using these approaches, CAD/CAM and biofabrication technologies have the potential to deliver custom-made implants with high shape-fidelity to the patient [33, 99, 364, 391].

Maintaining the shape

Biofabrication technologies can supply a highly controllable supporting scaffold by incorporating cell-containing hydrogels in a polymer scaffold. Robotic dispensing or inkjet printing principles allow deposition circumstances that can be tailored specifically to the various components of a hybrid construct, co-depositing hydrogels and thermoplastic polymer scaffolds with high spatial resolution (Figure 7) [281, 311]. Bioprinting permits optimization of the mechanical features of the construct, such as porosity, stiffness and strength, as both composition of the construct and the features of each component can easily be adjusted [81, 209]. Native auricular cartilage is a strong yet flexible tissue. Its tensile modulus, a measure of stiffness, has been reported to be approximately 16 MPa [190]. The ultimate tensile strength, the maximum stress a material can withstand, has been reported to be 2.18 MPa for native auricular cartilage [279]. In contrast, the tensile strength of hydrogels suitable for the encapsulation of cells is generally two to three orders of magnitude lower [89].

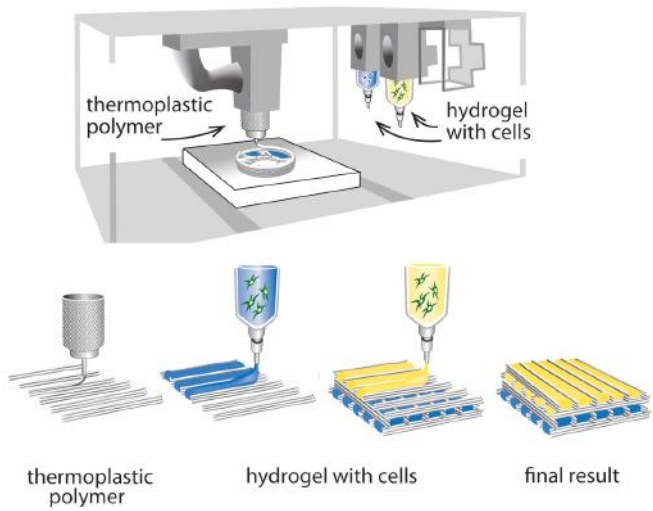

Figure 7. Highly controllable bioprinting of multiple materials. Bioprinting allows for the co-deposition of hydrogels and reinforcing polymer scaffolds with high spatial resolution. Hybrid constructs are fabricated in a layer-by-layer manner. Reprinted with permission and adapted from Schuurman *et al.* (2011) [311]. Reproduced with permission of IOP Publishing.

The strength provided by a scaffold is essential to maintain dimensions while the cells produce their extracellular matrix, until the newly formed tissue is strong enough to maintain itself and withstand the contractive forces of the skin. The mechanical strength can be increased by the density of crosslinks (either based on photo-, chemical, or thermal initiation) [54]. However, as high polymer crosslinking density and polymer content restrict cell proliferation and migration, the ideal hydrogel scaffold for biofabrication should preferably be composed of a lightly crosslinked bioink that at low concentrations still maintains printing accuracy (Figure 4). The stiffness of a hydrogel-only construct will be inferior to many native tissues, while auricular implants will face challenging contractive skin forces. With biofabrication techniques, biocompatible thermoplastic scaffold structures can be incorporated to increase their structural support. This consequently also allows the use of softer hydrogels as bioinks [311]. The ideal balance of hydrogel/polymer ratio will permit a suitable aqueous cellular microenvironment for cell growth and differentiation, as well as provide adequate strength for shape maintenance. Ultimately, the polymer support network will slowly degrade and be replaced by strong new tissue [25, 209, 311].

Maturation and remodeling of the new tissue is an extremely important factor contributing to the end result, as exposing constructs to contractive skin forces early in the maturation process may lead to deformation and degradation of the implant [27, 49, 209, 295, 389]. As nutrient limitation

causes central cell death, shortening the distance that nutrients have to travel can ensure all areas in a construct have access to sufficient nutrients. One option is to incorporate perfusion channels in the constructs so that during pre-culture nutrient-rich media is allowed access to the more inner parts of the construct. A modular approach (Figure 6), where the implant is made up of separate parts, may be another potential solution. The modules could be matured separately under more controlled conditions than larger engineered constructs can experience, and would be attached to one another once the neo-cartilage is strong enough for implantation under the skin.

Combining multiple tissues in the construct

The human external ear is a complex shape consisting of several tissue types. A normal ear consists of a cartilage framework, coated by a perichondrium layer, and then covered by the vascularized skin. Caudal of the cartilage framework is an earlobe consisting of fatty tissue. The auricular implant could consist of just the cartilage framework, and the ear reconstruction will be completed using skin flaps for the creation of the ear lobe. However, biofabrication does provide the opportunity to incorporate the fatty tissue earlobe into the implant [190], or even engineer a complete ear including the covering vascularized perichondrium and skin.

As pointed out earlier, one study used 3D printing technology to create a composite tissue in the shape of the human auricle, incorporating both chondrocytes and adipocytes for the regeneration of the cartilage framework and the fatty tissue earlobe, respectively [190]. The cells were printed separately in their respective locations within an ear-shaped polymer framework. Although the above-mentioned study demonstrated that co-fabrication of multiple tissues within one construct is technically feasible, the control and regulation of the simultaneous generation of multiple types of tissue in a single construct is still a challenge and further *in vitro* and *in vivo* analysis is required [190].

FUTURE PERSPECTIVES

This review addresses the various challenges in engineering a viable implant for auricular reconstruction. A first challenge for auricular implants is the design of the intricate shape and the subsequent maintenance of that shape. Biofabrication technologies are able to create complex 3D constructs with a highly detailed internal and external architecture. Auricular reconstruction is an aesthetic practice and, therefore, requires a personalized approach. Ultimately, the design of a restorative auricular implant should closely match the shape of the contralateral ear in order to achieve the best results. CAD/CAM technology has the potential to provide these patient-specific shapes for the design of the implant and can thus play an important role in personalized medicine approaches.

Biofabrication technologies also have the capacity to incorporate various materials into hybrid structures, including live cells, natural matrix components and reinforcing polymer fibers. The addition of bioactive cues, such as growth factors, or dECM to the cellular microenvironment can enhance growth and differentiation. The biofabrication of auricular cartilage implants is still in its infancy, and additional optimization of construct composition and structure is still required until conditions for routine clinical application are attained. As the insertion of artificial materials could elicit any degree of foreign body response or rejection by the immune system – causing inflammation and possible deformation of the construct or extrusion of the materials through the skin – the immunologic response to, as well as the carcinogenic potential of such materials, should be carefully evaluated before translation to the clinic.

An additional important issue in tissue engineering is the improvement of the accessibility of nutrients within constructs and the subsequent maturation of the neo-tissue. Although aberrant from normal cartilage tissue where the surrounding vascularized tissues are responsible for nutrient supply to the mature cartilage, a possible solution is the incorporation of a (temporary) engineered perfusion network within the construct. An alternative approach is the design of a modular construct, in which parts of the complete implant are matured separately. The design should result in modules that are accessible for nutrients by diffusion to ensure proper tissue maturation. Such auricular modules could then be matured separately in an *in vitro* and/or *in vivo* bioreactor [199]. Nevertheless, subsequent integration of the modules still needs to be addressed, as this has been shown to be dependent of the degree of maturation of the neocartilage tissue [222, 258].

Despite a maturation phase, the developing tissue will initially exhibit only limited mechanical strength. For auricular implants, however, initial mechanical integrity is of utmost importance as the contractive forces of the covering skin may cause degradation and deformation of the construct. To overcome this issue, cell-laden hydrogels can be reinforced with a polymer fiber network for increased mechanical strength. Such hybrid constructs exhibit increased mechanical strength as demonstrated by a higher Young's modulus and ultimate tensile strength [375]. Fiber reinforcement of hydrogels can be applied in a layer-by-layer fashion through multi-head robotic dispensing, inkjet printing, or organized microfiber deposition through electrospinning [311, 363, 375]. In order to select appropriate reinforcing polymers, extensive evaluation of the printability, cytocompatibility, degradation and (temporal) mechanical strength of candidate materials is required.

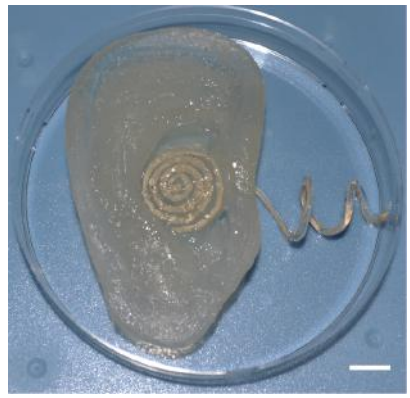

Figure 8. The bionic ear. Functional nanoelectrical components integrated within the tissue-engineered auricle. The bionic ear was able to perceive signals in the hertz to gigahertz range. Reprinted with permission from Mannoor *et al*. (2013) [212]. Copyright 2013 American Chemical Society.

The next step towards more complex tissue engineering in biofabrication is the co-deposition of multiple pre-tissue types within a single construct [192]. Although a biofabricated ear could consist of solely the auricular implant, it may also incorporate the fatty tissue earlobe [190], or even the covering perichondrium and skin. Engineering the auricle with its multiple tissue types and complex shape can be a step towards increased complexity in tissue engineering. Furthermore, the successful integration of functional nanoelectrical components within the biologically active engineered tissue (Figure 8) [212] further underscores the versatility and potential of biofabrication technologies towards creating more complex and functional structures, tissue parts or eventually whole organs.

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PART I

CELLS

FINDING AN APPROPRIATE CELL SOURCE
FOR ENGINEERING THE HUMAN AURICLE

CHAPTER 3

PROGENITOR CELLS IN AURICULAR CARTILAGE DEMONSTRATE CARTILAGE-FORMING CAPACITY IN 3D HYDROGEL CULTURE

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ABSTRACT

Paramount for the generation of auricular structures of clinically-relevant size is the acquisition of a large number of cells maintaining an elastic cartilage phenotype, which is the key in producing a tissue capable of withstanding forces subjected to the auricle. Current regenerative medicine strategies utilize chondrocytes from various locations or mesenchymal stromal cells (MSCs). However, the quality of neo-tissues resulting from these cell types is inadequate due to inefficient chondrogenic differentiation and endochondral ossification, respectively. Recently, a subpopulation of stem/progenitor cells has been identified within the auricular cartilage tissue, with similarities to MSCs in terms of proliferative capacity and cell surface biomarkers, but their potential for tissue engineering has not yet been explored. The current study compared the *in vitro* cartilage-forming ability of equine auricular cartilage progenitor cells (AuCPCs), bone marrow-derived MSCs and auricular chondrocytes in gelatin methacryloyl (gelMA)-based hydrogels over a period of 56 days, by assessing their ability to undergo chondrogenic differentiation. Neocartilage formation was assessed through gene expression profiling, compression testing, biochemical composition and histology. Similar to MSCs and chondrocytes, AuCPCs displayed a marked ability to generate cartilaginous matrix, although, under the applied culture conditions, MSCs outperformed both cartilage-derived cell types in terms of matrix production and mechanical properties. AuCPCs demonstrated upregulated mRNA expression of elastin, low expression of collagen type X and similar levels of proteoglycan production and mechanical properties as compared to chondrocytes. These results underscored the AuCPCs' tissue-specific differentiation potential, making them an interesting cell source for the next generation of elastic cartilage tissue-engineered constructs.

BACKGROUND

A significant challenge in cartilage tissue engineering is the recruitment of a sufficient number of cells for the generation of large tissue constructs [25]. The necessity for larger constructs is exemplified by the case of auricular reconstruction required for the congenital disorder microtia and for defects of the auricle caused by injury and disease. For the human auricle, estimates of the number of cells required for tissue regeneration range between 100 and 150 million [25], with the obvious challenge of obtaining this number of cells from an autologous source.

Although considerable progress has been made using a variety of cells for auricular cartilage tissue engineering, there is no definitive conclusion on which cell type is capable of providing the most favorable clinical outcome for tissue-engineered cartilage constructs. Originating from the native tissue, chondrocytes are a logical cell source for the generation of neocartilage. Auricular [26, 49, 116, 155, 185, 240, 271, 279, 294, 295, 306, 349, 373], microtia [153, 169, 240], nasoseptal [11, 27, 136, 155, 185], costal [155, 185] and articular chondrocytes [155, 168, 185, 224] are all used for tissue-engineering auricular cartilage. Specific characteristics and ability for chondrogenesis can differ depending on the origin of the chondrocytes. Where the articular and costal cartilage are of mesenchymal origin, the developmental origin of the auricular cartilage of the pinna is still controversial. The auricle originates from two pharyngeal arches that have contributions from all three embryonic layers [128, 203, 347, 371]. Nevertheless, fully differentiated cells from various cartilage origins generally demonstrate the capacity to produce cartilage-like extracellular matrix *in vitro* and *in vivo* [2, 27, 155, 185, 292]. However, only a small amount of tissue can be harvested from the patient, requiring extensive *in vitro* expansion of the isolated cells to obtain a sufficient cell's number for the production of clinically-relevant tissue-engineered cartilage constructs. The proliferative potential of chondrocytes is naturally low [288] and their cartilage-forming ability is known to decline with extended cultivation [144, 307]. In fact, repeated passaging of chondrocytes induces the loss of their chondrogenic phenotype [303] and, ultimately, leads to progressive dedifferentiation. To some extent, re-differentiation can be attained under specific culture conditions, *e.g.* in three-dimensional (3D) environments [19] and in the presence of appropriate growth factors [156, 355], yet the revenue is limited [355]. The resulting neo-tissue is often of a fibrocartilagenous quality and exhibits inferior biochemical and mechanical properties as compared to native cartilage tissue [63, 80, 234, 368]. In the past decade, stem cells have gained increasing interest for tissue engineering applications because of their capacity for self-renewal and multilineage differentiation. For example, bone-marrow-derived mesenchymal stromal cells (MSC) can be extensively expanded [117] and demonstrate the ability to differentiate into various cell types, including chondrocytes, osteoblasts and adipocytes [290]. Numerous studies have successfully used MSCs for the generation of cartilage [60, 313, 359], sometimes in co-culture with auricular chondrocytes [200, 291, 387]. Nevertheless, the risk of hypertrophic growth, terminal

differentiation and subsequent tissue calcification remains an important limitation when using MSCs for cartilage tissue engineering [120, 368]. In addition, bone marrow-derived MSCs are not naturally involved in the development of native auricular cartilage and are, as articular, costal and nasoseptal chondrocytes, not predisposed to produce the elastic fibers required for this elastic-type cartilage [185].

Tissue-derived stem/progenitor cells exhibit stem-cell-like qualities, such as self-renewal and multipotency, yet are embedded within the target tissue in niches and are primed to differentiate to that tissue [157]. The identification of resident progenitor cell populations in articular cartilage [86, 368] as well as in auricular and tracheal perichondrium [82, 180, 346] has opened up new pathways for cartilage tissue engineering. Like MSCs, which can undergo up to 70 population doublings [62], cartilage progenitor/stem cells retain proliferative ability for up to 60 population doublings [368], demonstrating potential for accumulating large cell numbers starting from a single cell. In addition, the cells maintain multipotent differentiation ability while expressing chondrocyte-specific characteristics [215]. It is hypothesized that these tissue-specific progenitor cells are highly primed to differentiating into the chondrogenic lineage [157, 215].

Auricular cartilage tissue engineering may benefit greatly from a source of tissue-specific cells that can be expanded up to large numbers without losing their differentiation potential. Nevertheless, the auricular cartilage itself was, until recently, unknown to harbor a progenitor cell population. The presence of colony-forming, multipotent progenitor cells in auricular cartilage has recently been confirmed [377], though these cells have not yet been explored for a tissue-engineering purpose. The objective of the current study was to evaluate the cartilage-forming ability of such auricular cartilage progenitor cells in a 3D hydrogel culture. It was hypothesized that these cells, despite *in vitro* expansion, could outperform auricular chondrocytes and bone-marrow-derived MSCs with regards to production of auricular cartilage.

Mimicking the hydrated environment of native cartilage, hydrogels are particularly attractive for cartilage regenerative strategies. Gelatin methacryloyl (gelMA)-based hydrogels are a versatile group of biomaterials shown to facilitate cartilage-like matrix production for chondrocytes, articular cartilage progenitor cells as well as MSCs, supporting both cell viability and mechanical properties [77, 113, 193, 312]. In this study, cell-laden gelMA hydrogels were cultured for up to 8 weeks in chondrogenic differentiation media and harvested at day 1, 28 and 56 for mechanical testing, biochemistry, gene expression, histology and immunohistochemistry for the assessment of cartilage-specific properties.

MATERIALS & METHODS

Isolation of cells

Primary auricular chondrocytes (AuCH) and auricular cartilage progenitor cells (AuCPC) were obtained from deceased equine donors, which were kindly provided by a local slaughterhouse. Bone-marrow-derived MSCs were obtained from healthy equine donors. All tissues and cells were obtained according to the guidelines of the Institutional Animal Ethical Committee (The Netherlands).

AuCHs and AuCPCs were harvested from the auricles of fresh equine cadavers (3- to 10-year-old; $n = 3$). The ears were cut off at the base, shaved, thoroughly washed with soap and soaked for circa 15 minutes in Betadine® (Meda Pharma, The Netherlands). Under sterile conditions, an incision through the skin was made on the dorsal side along the longitudinal axis and the skin and subcutaneous tissue were dissected. Ensuring the harvest of auricular cartilage exclusively, the perichondrium was fully removed by carefully scraping the tissue off with surgical tools. Cartilage chips were sectioned off the scapha of the ear, washed in sterile phosphate-buffered saline (PBS) and, subsequently, minced into 1 mm^2 pieces. The tissue was digested in 0.2 % pronase (Roche, USA) for 2 hours followed by 16 hours in 0.075 % collagenase type II (Worthington Chemical Corporation, USA) digestion at 37 °C. Next, the solution was filtered through a 70 µm cell strainer and centrifuged for 5 minutes at 300 ×*g* to obtain a cell pellet. AuCHs were washed in sterile PBS, counted with a hemocytometer and stored at passage 0 in liquid nitrogen until further use.

For each donor, an aliquot of the freshly-isolated cells was saved for the isolation of AuCPCs, for which the cells were subjected to a fibronectin adhesion assay as previously described [86, 368]. Briefly, cells suspended in serum-free Dulbecco's modified Eagle medium (DMEM; 31966, Gibco, USA) were plated at a density of 500 cells/cm^2 on fibronectin-coated tissue culture plates. After 20 minutes of incubation at 37 °C, the non-adherent cells were carefully removed. Attached progenitor cells were cultured in chondroprogenitor expansion media, consisting of DMEM supplemented with 10 % v/v fetal bovine serum (FBS; Lonza, USA), 0.2 mM L-ascorbic acid 2-phosphate (Sigma-Aldrich, The Netherlands), 100 U/mL penicillin (Life Technologies, USA), 100 µg/mL streptomycin (Life Technologies) and 5 ng/mL basic fibroblast growth factor (bFGF; Peprotech, UK). After 6 days of culture, colonies consisting of >32 cells were harvested. Monoclonal colonies were pooled and expanded until passage 3, when they were stored in liquid nitrogen until further use.

MSCs were obtained from bone marrow aspirates from the sternum of healthy equine donors (3- to 10-year-old; $n = 3$) and the mononuclear fraction was isolated following a previously described protocol using a Ficoll®-Paque density gradient (GE Healthcare, The Netherlands) [362]. After isolation, MSCs were cultured in MSC expansion medium, consisting of alpha modification

minimum essential medium (αMEM) (22561, Gibco) supplemented with 0.2 mM L-ascorbic acid 2-phosphate (Sigma-Aldrich), 10 % FBS (Lonza), 100 U/mL penicillin (Life Technologies), 100 µg/mL streptomycin (Life Technologies) and 1 ng/mL bFGF (Peprotech) until passage 3 and subsequently stored in liquid nitrogen until further use.

Characterization of equine auricular chondroprogenitor cells

AuCHs (at passage 1), AuCPCs and MSCs (both at passage 3) were characterized by comparison of gene expression of cell surface markers and assessment of multilineage differentiation potential in two-dimensional (2D) culture.

The expression of cell membrane markers was evaluated in duplicate by a reverse transcriptase-polymerase chain reaction (RT-PCR), comparing the transcriptome of AuCHs, AuCPCs and MSCs. Analyzed target genes included *CD13*, *CD29*, *CD31*, *CD34*, *CD44*, *CD45*, *CD49d*, *CD73*, *CD90*, *CD105*, *CD106*, *CD146* and *CD166*, which were compared to expression of the housekeeping gene hypoxanthine phosphoribosyltransferase (*HPRT1*). Primer sequences and expected amplicon sizes are reported in Supplementary Table 1. RNA was isolated using the RNeasy mini kit (Qiagen, Germany), according to the manufacturer's instructions. Isolated mRNA was quantified by UV-vis spectrophotometry with a Nanodrop 2000 (Thermo Scientific, The Netherlands) to serve as template for polymerase chain reaction (PCR). The amplification of RNA was carried out using a SuperScript® One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Life Technologies) and the PCR products were run on agarose gel stained with ethidium bromide. Subsequently, the amplicons were imaged using a UV transilluminator (ProXima 10 Phi; Isogen Life Sciences, The Netherlands).

Multipotency was evaluated in duplicate through an *in vitro* trilineage differentiation assay in which cells were directed towards bone, fat or cartilage by culturing them in osteogenic, adipogenic or chondrogenic differentiation media, respectively. For osteogenic and adipogenic differentiation, cells were plated in 6-well culture plates at a density of 2×10^5 cells/well and cultured in chondroprogenitor expansion medium until sub-confluency, before initiating differentiation. Osteogenic differentiation medium consisted of αMEM (Gibco) supplemented with 10 % v/v FBS (Lonza), 100 U/mL penicillin (Life Technologies), 100 µg/mL streptomycin (Life Technologies), 0.2 mM L-ascorbic acid 2-phosphate (Sigma-Aldrich), 20 mM β-glycerol phosphate (Sigma-Aldrich) and 100 nM dexamethasone (Sigma-Aldrich). Adipogenic medium consisted of αMEM (Gibco) supplemented with 10 % v/v FBS (Lonza), 100 U/mL penicillin (Life Technologies), 100 µg/mL streptomycin (Life Technologies), 0.01 mM indomethacin (Sigma-Aldrich), 83 mM 3-Isobutyl-1-metylxanthine (Sigma-Aldrich) and 1.72 µm bovine pancreas-derived insulin (Sigma-Aldrich). Culture medium was refreshed every 3 days. For chondrogenic differentiation, 2.5×10^5 cells were pelleted by centrifugation at 300 ×*g* in 15 mL Falcon® tubes. Subsequently, the pellets were

cultured in chondrogenic differentiation medium, consisting of DMEM supplemented with 1 % v/v insulin-transferrin-selenous acid (ITS+ Premix; Corning, USA), 0.2 mM L-ascorbic acid 2-phosphate (Sigma-Aldrich), 100 U/mL penicillin (Life Technologies), 100 µg/mL streptomycin (Life Technologies), 100 nM dexamethasone (Sigma-Aldrich) and 10 ng/mL transforming growth factor β1 (TGF-β1; Peprotech). After 14 days of culture, osteogenic commitment was evaluated by Alizarin Red S staining to observe calcified matrix deposition, whereas adipogenic differentiation was assessed with Oil Red O staining to visualize the formation of intracellular lipid vesicles. Cell pellets were embedded in paraffin and 5 µm-thick sections were stained with Safranin O to visualize glycosaminoglycans (GAG), indicative of chondrogenic differentiation.

Fabrication of cell-laden hydrogel constructs for 3D culture

Following a previously published protocol [218], the hydrogel gelatin methacryloyl (gelMA) was synthesized by functionalizing gelatin type A (obtained from porcine skin; Sigma-Aldrich) in PBS with methacrylic anhydride groups, to obtain a hydrogel with a 80 % degree of functionalization. Subsequently, a 10 % w/v solution of gelMA was supplemented with 0.1 % w/v 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959; BASF, Germany) as a photoinitiator. AuCHs, AuCPCs or MSCs, expanded beforehand until passage 1, 4 and 4, respectively, were homogeneously suspended in the hydrogel at 37 °C at a concentration of 1.5×10^7 cells/mL. The cell-laden gel was immediately casted into a custom-made Teflon™ mold to produce cylindrical samples (diameter = 6 mm, height = 2 mm) and subsequently chemically crosslinked by UV irradiation for 5 minutes (wavelength λ = 365 nm, intensity E = 3 mW/cm^2, at a height of 2 cm; 144 portable UV lamp, Vilber Lourmat, Germany), to trigger free-radical polymerization. Cell-free hydrogel samples (3 replicates per timepoint) were used as controls and were prepared following the same steps. All hydrogel samples were cultured in chondrogenic differentiation medium for 1, 28 and 56 days at 37 °C and 5 % CO_2, refreshing media 3 times per week.

Gene expression of cartilage markers after chondrogenic differentiation

The relative gene expression of cartilage markers in cell-laden hydrogels (n = 3) was evaluated by qPCR at 1 and 56 days of culture. Analyzed markers included aggrecan (*ACAN*), cartilage oligomeric matrix protein (*COMP*), collagen type II (*COL2A2*), collagen type I (*COL1A1*), collagen type X (*COLXA1*), runt-related transcription factor 2 (*RUNX2*) and elastin. The expression levels were normalized against the housekeeping gene *HPRT1*. Primer sequences for each transcript are reported in Supplementary Table 2.

Gel samples were mechanically ground in RLT buffer (Qiagen). From the lysate, mRNA was isolated using the RNeasy Mini Kit (Qiagen) and subsequently quantified with a Nanodrop 2000 (Thermo Scientific). A SuperScript® III Platinum SYBR Green One-Step qRT-PCR Kit (Life Technologies) was used for amplification of the mRNA and cDNA synthesis, which was performed with a LightCycler®

96 (Roche). The PCRminer algorithm was used to calculate relative gene expression, Ct and efficiency values [386].

Biochemical analysis of cell-laden hydrogels

After 1, 28 and 56 days in culture, 4-6 replicates of each group of cell-laden hydrogels were taken for quantification of DNA and GAG content. Cell-laden hydrogel samples were frozen at –20 °C and subsequently lyophilized. The wet and dry weights were recorded during this process. Digestion of samples occurred overnight at 60 °C in 200 µL papain digestion buffer (P3125; Sigma-Aldrich), consisting of 0.2 M NaH_2PO_4 (Merck, USA) and 0.01 M ethylenediaminetetraacetic acid (EDTA; VWR, USA) in milliQ water (pH = 6.0) supplemented with 250 µL/ mL papain solution (16-40 units/ mg of protein) and 0.01 M cysteine (C9768; Sigma-Aldrich).

Total DNA content was quantified using a Quant-iT PicoGreen dsDNA assay (Life Technologies) and compared to a standard of known concentrations of DNA. The fluorescence was measured at 485 nm excitation and 520 nm emission by a spectrofluorometer (Bio-Rad, USA).

Total GAG content, as a measure of cartilage-specific matrix production, was quantified using a dimethylmethyleneblue (DMMB; pH = 3.0) assay. The 525/595 nm absorbance ratio was measured with a VersaMax plate reader (Molecular Devices, UK). The sulfated GAG (sGAG) content was calculated using a standard of known concentrations of chondroitin sulfate C and corrected for the dilution factor.

Both dsDNA and sGAG content were normalized against dry weight. The ratio of GAGs per DNA was calculated to display the activity of single cells in producing cartilage-specific matrix.

Compressive mechanical testing of hydrogel constructs

An unconfined uniaxial compression test was performed to evaluate the mechanical properties of the cell-laden hydrogel samples after 1, 28 and 56 days in culture (4-6 replicates per timepoint). Using a dynamic mechanical analyzer (DMA Q800; TA Instruments, Belgium), samples were compressed at a –20 %/min strain rate to –30 %. The compressive Young's modulus was calculated as the slope of the initial linear segment (10-15 % strain) of the stress/ strain curve.

Histology and immunohistochemistry

Deposition of the main components of cartilage extracellular matrix in cell-laden hydrogels were visualized by histology and immunohistochemistry on formalin-fixed, paraffin-embedded samples. After 1, 28 and 56 days in culture, samples from each group were fixated in 4 % neutral buffered formalin. Samples were then dehydrated through a graded ethanol series (70 %, 96 %, 100 % ethanol), cleared in xylene and subsequently embedded in paraffin. The samples were sectioned into 5 µm slices, and deparaffinized prior to staining. For the identification of cartilage

glycosaminoglycan deposition, a triple stain consisting of hematoxylin (cell nuclei), Fast Green (collagens) and Safranin O (proteoglycans) was applied. Deposition of collagens was evaluated by immunohistochemistry, with appropriate primary antibodies for collagen type II (II-II6B3; DSHB, USA), collagen type I (sc-8784; Santa Cruz Biotechnology, USA) and collagen type VI (5C6; DSHB). In addition, appropriate IgG were used as isotype controls. After deparaffinization, samples were treated with 0.3 % v/v H_2O_2 to block endogenous peroxidases. Antigen retrieval was performed with pronase (1 mg/mL; Roche) and hyaluronidase (10 mg/mL; H2126, Sigma-Aldrich), both applied for 30 minutes at 37 °C. Subsequently, tissue sections were blocked with bovine serum albumin (BSA; 5 % w/v in PBS) for 1 hour at room temperature. The primary antibodies were incubated overnight at 4 °C, followed by an HRP-tagged secondary antibody for 1 hour at room temperature before the staining was developed with 3,3-diaminobenzidine-horseradish peroxidase (Sigma-Aldrich). Cell nuclei were counterstained with hematoxylin. A Von Kossa staining was applied to detect calcium precipitates indicating tissue mineralization. All stained sections were mounted in DPX (Millipore, USA) and examined using a light microscope (Olympus BX51; Olympus, Germany).

Statistical analysis

Quantitative results are expressed as mean ± standard error of the mean (SEM). The means of the experimental groups were compared at different timepoints by performing a two-way ANOVA with a Bonferroni post-hoc test. The statistical analyses were performed using Graphpad Prism 7 (Graphpad Software, USA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Equine auricular cartilage progenitor cells display stem cell qualities

Histological evaluation confirmed that the perichondrial layer attached to auricular cartilage was successfully removed and it was therefore assumed that a population of exclusively cartilage-derived cells was obtained after tissue digestion, prior to isolation of chondroprogenitors using differential adhesion to fibronectin (Figure 1).

Gene expression of several surface markers was analyzed for isolated AuCHs, AuCPCs and MSCs by RT-PCR (Figure 2A), demonstrating that the transcript profile of AuCPCs shared similarities with both AuCHs and MSCs. Like MSCs, AuCPCs were positive for the stem cell markers *CD73, CD90* and *CD105*, and negative for the hematopoietic marker *CD34* and for the leukocyte marker *CD45*. Thus, at the transcript level, AuCPCs satisfied the minimal requirements for classification of human MSCs [84]. Furthermore, all three cell types were $CD29^+$, $CD31^+$, $CD106^+$ and $CD166^+$. Differences between cell types arose in the expression of *CD13, CD49d* and *CD146*. *CD13* was highly positive in AuCPCs, to a lesser extent in MSCs, and negative in AuCHs. *CD49d* appeared positive for MSCs and AuCPCs, and negative for AuCHs. Lastly, *CD146* was faintly positive in both MSCs and AuCPCs, and negative in AuCHs.

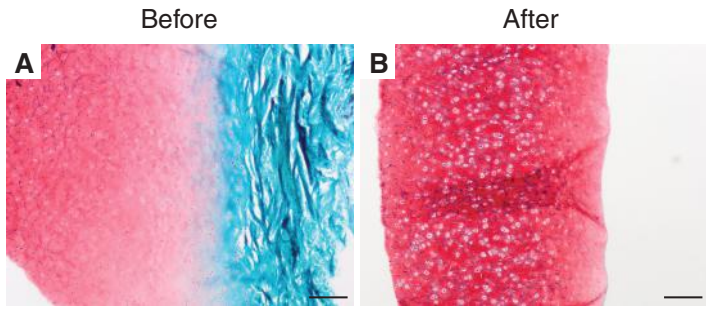

Figure 1. Histological analysis demonstrating successful perichondrium removal. Safranin O staining on auricular cartilage before (A) and after (B) utilizing the scraping method for the removal of perichondrium, confirming its complete removal from the auricular cartilage. Scale bars equal 200 µm.

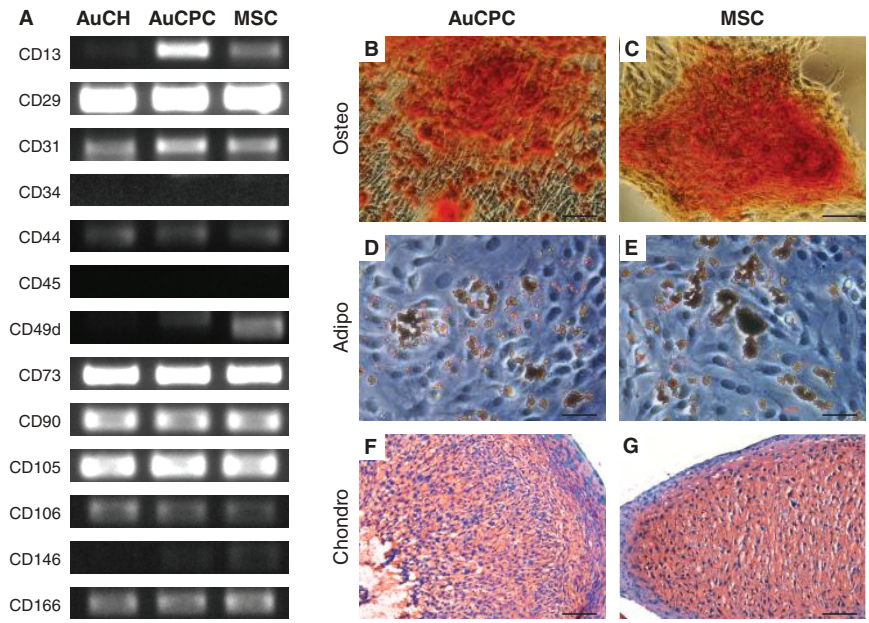

Figure 2. Characterization of AuCPCs in comparison to AuCHs and MSCs. The expression of several surface markers, obtained from RT-PCR, was compared amongst the cell types (A). Trilineage differentiation of AuCPCs and MSCs demonstrated positive Alizarin Red staining for osteogenic (B/C), Oil Red O staining for adipogenic (D/E) and Safranin O staining for chondrogenic (F/G) differentiation. Scale bars equal 100 µm.

Multipotency of AuCPCs was assessed through a trilineage differentiation assay. Like MSCs (Figure 2C, 2E, 2F), AuCPCs demonstrated to be capable of differentiating towards bone (Figure 2B), adipose tissue (Figure 2D) and cartilage (Figure 2F), confirming their multipotent potential.

Differential mRNA expression of cartilage markers in hydrogel culture

As measured by qPCR, AuCHs, AuCPCs and MSCs embedded in 3D hydrogels demonstrated increased relative-fold expression levels of cartilage-specific gene transcripts over time, confirming their differentiation in the hydrogel (Figure 3).

Aggrecan expression (Figure 3A) after 56 days was highest in AuCHs (48.8-fold upregulation), followed by AuCPCs (37.7-fold) and finally MSCs (15.7-fold). The same correlation was already apparent at day 1 of culture: mRNA expression of aggrecan was significantly more upregulated in AuCHs and AuCPCs (10.8- and 8.6-fold respectively) than MSCs, expressing a 0.1-fold reduction. The expression of aggrecan increased over time in all three cell types, yet this increase was significant only for AuCHs.

A similar relation was observed for the mRNA expression of *COMP* (Figure 3B), which is the most abundant non-collagenous matrix protein present in cartilage. AuCPCs exhibited a significantly higher expression level than MSCs after 56 days of culture (186.2-fold in AuCPCs versus 64.2-fold in MSCs), with AuCHs displaying a 146.7-fold increment. Likewise, AuCPCs already demonstrated the highest upregulation of *COMP* (39.1-fold) at day 1, followed by AuCHs (20.5-fold) and for MSCs only 0.9-fold relative to the housekeeping gene. Both AuCHs and AuCPCs displayed a significant increase in expression levels over time, whereas MSCs did not.

After 56 days of culture, AuCHs and AuCPCs expressed *COL2A1* (Figure 3C) at comparable levels, with a 51- and 60-fold increment, respectively. MSCs exhibited the highest levels of *COL2A1* mRNA with an 89.1-fold increment at day 56, yet this difference was not significant. Expression of *COL2A1* was considerably lower (<3.5-fold) in all three cell types at the beginning of culture, indicating a significant increase over time in the case of AuCPCs and MSCs. Conversely, the expression of *COL1A1* (Figure 3D) was already highly upregulated at the beginning of culture in MSCs (59.9-fold increment), compared to a significantly lower 19.3-fold in AuCHs and 22.8-fold in AuCPCs. This trend reversed during chondrogenic differentiation, as AuCHS significantly increased the expression of *COL1A1* over time. Differences between cell types were not significant at day 56, yet the highest expression at day 56 in AuCHs (77.2-fold increment), followed by equivalent levels in AuCPCs and MSCs (54.2- and 48.1-fold respectively).

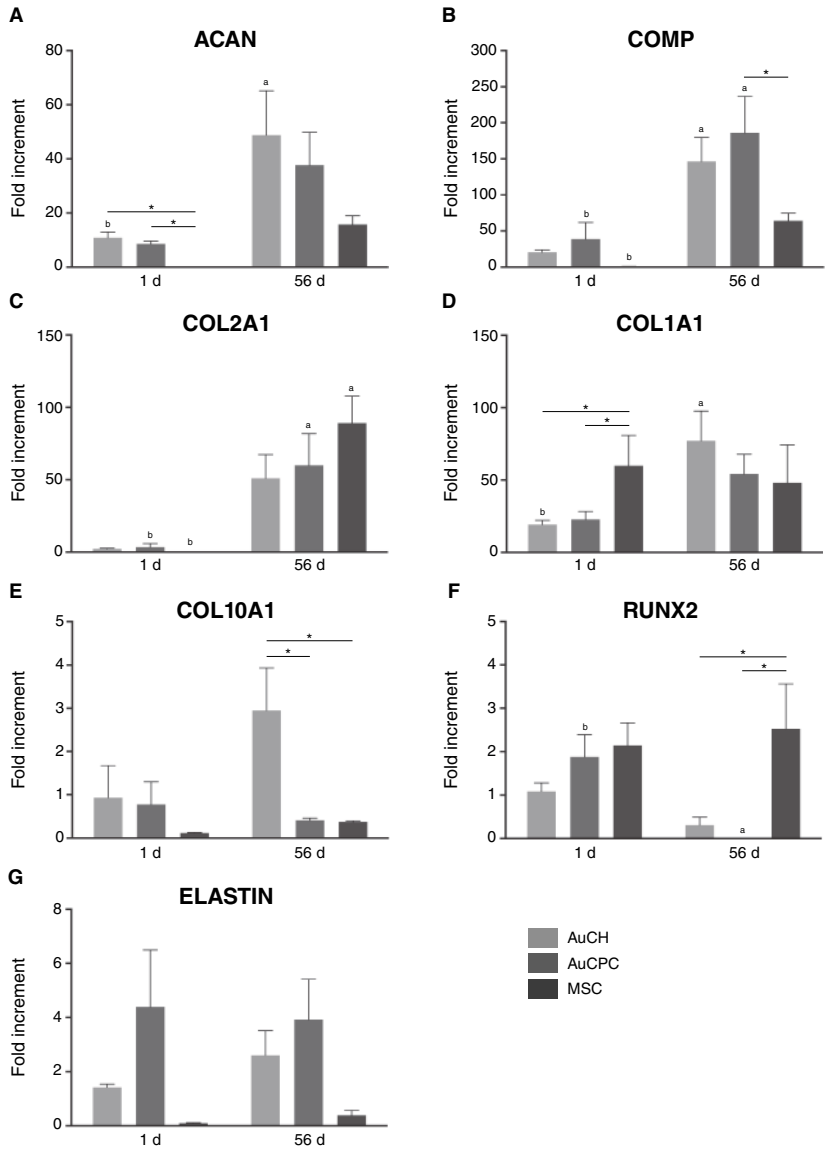

Figure 3. Cartilage-specific markers are upregulated in AuCPCs. Relative gene expression of aggrecan (*ACAN*) (A), cartilage oligomeric matrix protein (*COMP*) (B), collagen type II (*COL2A1*) (C), collagen type I (*COL1A1*) (D), collagen type X (*COL10A1*) (E), runt-related transcription factor 2 (*RUNX2*) (F) and elastin (G), as obtained from qPCR analysis of cell-laden hydrogels. Statistically significant differences of $p < 0.05$ for comparisons between cell types are marked with an asterisk (*). For each cell type, statistically significant differences of $p < 0.05$ are marked with [a] for a significant difference to day 1 and [b] for a significant difference to day 56.

AuCHs exhibited significantly higher values of *COL10A1* (Figure 3E) at the end of culture (3-fold) in comparison to both AuCPCs and MSCs, which both exhibited a 0.4-fold lesser expression than the housekeeping gene. AuCHs and MSCs both showed increasing yet non-significant trends in expression levels, whereas *COL10A1* expression levels by AuCPCs decreased non-significantly over time. The expression of *RUNX2* (Figure 3F), the master transcription factor in osteogenesis and endochondral ossification, was significantly higher in MSCs (2.5-fold) compared to AuCHs (0.3-fold) and AuCPCs (0.0-fold) at day 56 of culture. At the start of culture, *RUNX2* was upregulated by AuCHs by 1.1-fold, AuCPCs by 1.9-fold, and MSCs by 2.1-fold. AuCPCs demonstrated a significant decrease in its *RUNX2* expression over time to virtually zero at day 56.

The critical structural component of elastic cartilage is elastin. AuCHs exhibited higher mRNA expression of elastin (1.4-fold at day 1 and 2.6-fold at day 56) than MSCs (0.1-fold relative expression at day 1 and 0.4-fold at day 56). Although not significant, AuCPCs demonstrated the highest upregulation of elastin, with 4.4-fold and 3.9-fold increments at day 1 and 56 respectively (Figure 3G).

Chondrogenic differentiation in hydrogels results in cartilage-specific matrix deposition

Cell proliferation and extracellular matrix deposition in cell-laden hydrogels was assessed by quantification of dsDNA and sGAG content, representative of cell number and proteoglycan quantity respectively. As indicated by the increase in dsDNA content in the first 28 days of culture, AuCPCs and MSCs showed an ability to proliferate in the 3D gelMA hydrogel environment. The dsDNA content of AuCHs was significantly higher than AuCPCs and MSCs in the beginning of culture and stayed stable in the first 28 days, then significantly decreased to levels comparable to the other two cell types. These differences in cell number between groups at the beginning of culture could possibly be attributed to cell-loading inconsistencies. Nevertheless, dsDNA content among groups appeared to equalize over time (Figure 4A).

The synthesis of neocartilage matrix was evaluated by the amount of sulphated GAGs present in the hydrogel samples. AuCHs, AuCPCs and MSCs all produced significantly increasing amounts of sGAG over the course of chondrogenic culture, and these ECM components were retained in the hydrogel matrix. AuCHs and AuCPCs showed similar trends in total sGAG content (Figure 4B) as well as in sGAG normalized to the dsDNA content (Figure 4C). After 56 days of culture, AuCHs averaged 409.9 ± 35.7 mg/mg and AuCPCs 458.4 ± 30.9 mg/mg total sGAG content, and 509.9 ± 56.8 mg/mg and 565.2 ± 40.8 mg/mg sGAG/dsDNA respectively. MSCs initially appeared to lag behind in total sGAG content (214.5 ± 25.1 mg/mg at day 28, compared to 292.7 ± 29.5 for AuCHs and 308.3 ± 15.8 mg/mg for AuCPCs), yet encompassed lower cell numbers in the samples. Accordingly, when normalized to dsDNA content, AuCHs, AuCPCs and MSCs performed similarly at the 28-day timepoint (304.9 ± 42.6, 329.7 ± 19.9 and 296.9 ± 38.1 mg/mg respectively). After 56

days of culture however, when dsDNA levels were equivalent between groups, MSCs (789.5 ± 95.4 mg/mg) significantly outperformed both cartilage-derived cell types, displaying sGAG/dsDNA values 1.6-fold higher than AuCHs (508.9 ± 56.8 mg/mg) and 1.4-fold higher than AuCPCs (565.2 ± 40.8 mg/mg).

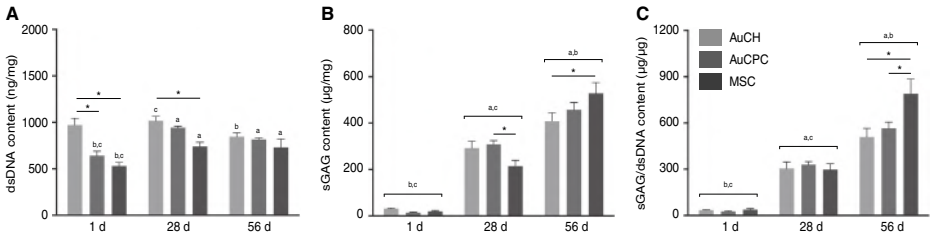

Figure 4. Analysis of biochemical composition in cell-laden hydrogels. Quantification of sulfated GAG content (A) and dsDNA content (B), as well as the sGAG per dsDNA ratio (C) in cell-laden hydrogels after 28 and 56 days of chondrogenic culture, all normalized against dry weight. Statistically significant differences of $p < 0.05$ for comparisons between cell types are marked with an asterisk (*). For each cell type, statistically significant differences of $p < 0.05$ are marked with [a] for a significant difference to day 1, [b] for a significant difference to day 28, and [c] for a significant difference to day 56.

Mechanical properties of cell-laden hydrogels increase over time

Compressive mechanical testing was performed to evaluate the progressive changes in the stiffness of the constructs over time (Figure 5). After 28 days of chondrogenic culture, the compressive Young's modulus of AuCHs (41.3 ± 3.0 kPa) and MSCs (44.9 ± 5.6 kPa) increased non-significantly with 1.6-fold and 1.8-fold respectively in comparison to cell-free samples. Both cell types were outperformed by AuCPCs (83.2 ± 8.1 kPa), which exhibited a significant 3.3-fold increase at this timepoint. When comparing these results to the total sGAG contents per sample, there is indeed a 1.4-fold difference between AuCPCs and MSCs; however, the observed difference in mechanical properties between AuCPCs and AuCHs is not reflected in total sGAG content.

At the end of the culture period, MSCs (179.2 ± 22.2 kPa) significantly outperformed both AuCHs (102.8 ± 10.2 kPa) and AuCPCs (108.6 ± 10.1 kPa) in terms of compressive Young's modulus (1.7-fold and 1.6-fold difference respectively), correlating with the observed trends in total sGAG and sGAG/dsDNA content. In comparison with cell-free hydrogels, cell-laden constructs demonstrated a significant increase in compressive modulus of 10.5-fold for MSCs, 6.4-fold for AuCPCs, and 6.1-fold for AuCHs.

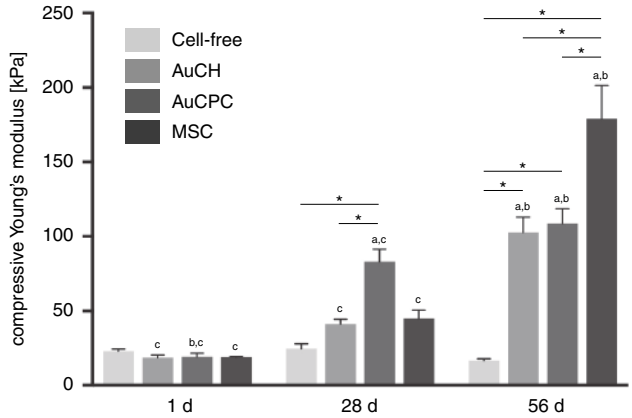

Figure 5. Mechanical properties of cell-laden hydrogels increase over time. Compressive Young's modulus of cell-laden hydrogels after 28 and 56 days of culture compared to cell-free samples. Statistically significant differences of $p < 0.05$ for comparisons between cell types are marked with an asterisk (*). For each cell type, statistically significant differences of $p < 0.05$ are marked with [a] for a significant difference to day 1, [b] for a significant difference to day 28, and [c] for a significant difference to day 56.

The compressive Young's modulus of cell-free constructs remained in the range of 16-25 kPa at all timepoints, indicating no notable degradation of the bulk properties of the hydrogel during the period of *in vitro* culture. In general, cartilage matrix synthesis in cell-laden hydrogels, as indicated by the total sGAG content, correlated with an increase in mechanical properties of the samples.

Histology and immunohistochemistry confirm cartilage-like matrix deposition

Histological sections displayed the presence and distribution of several main components of cartilage extracellular matrix in the hydrogel, including proteoglycans as well as collagen type II, I and VI (Figure 6).

AuCHs, AuCPCs and MSCs all demonstrated inhomogeneous distribution of synthesized proteoglycans throughout the hydrogel constructs (Figure 6A-C), with a gradation of decreasing labeling from the pericellular territorial to inter-territorial matrices. In all cases, there is an evident increase in the intensity of Safranin O staining for proteoglycans over time. In line with trends in total sGAG content, MSCs qualitatively displayed less proteoglycans at 28 days of culture, yet exhibited the most intense staining at the end of the culture period in comparison to AuCPCs and to a greater extent when compared to AuCHs. In all experimental groups, the deposition of collagen type II and type I seemed to occur predominantly in the outer rim of the cylindrical hydrogel constructs, with lighter staining in the center. The diameter of this outer rim appeared to widen over time, indicating increased matrix deposition towards the center of the construct.

MSCs and AuCPCs exhibited a more homogeneous distribution of collagen type II, whereas AuCHs displayed clusters of intense pericellular labeling (Figure 6D-F). A similar trend was noted in collagen type I (Figure 6G-I), where rims with the highest intensity staining were found in AuCH samples, followed by MSCs and AuCPCs. These observations correlate with *COL1A1* mRNA expression profiles at day 56. Collagen type VI-labelled clusters (Figure 6J-L) were observed pericellularly after 56 days of culture in AuCPCs and to a lesser extent in MSCs, with only sporadic staining in AuCHs. Finally, Von Kossa staining for mineralization did not show any black staining indicative of calcium in either group (Figure 6M-O), unlike the positive control.

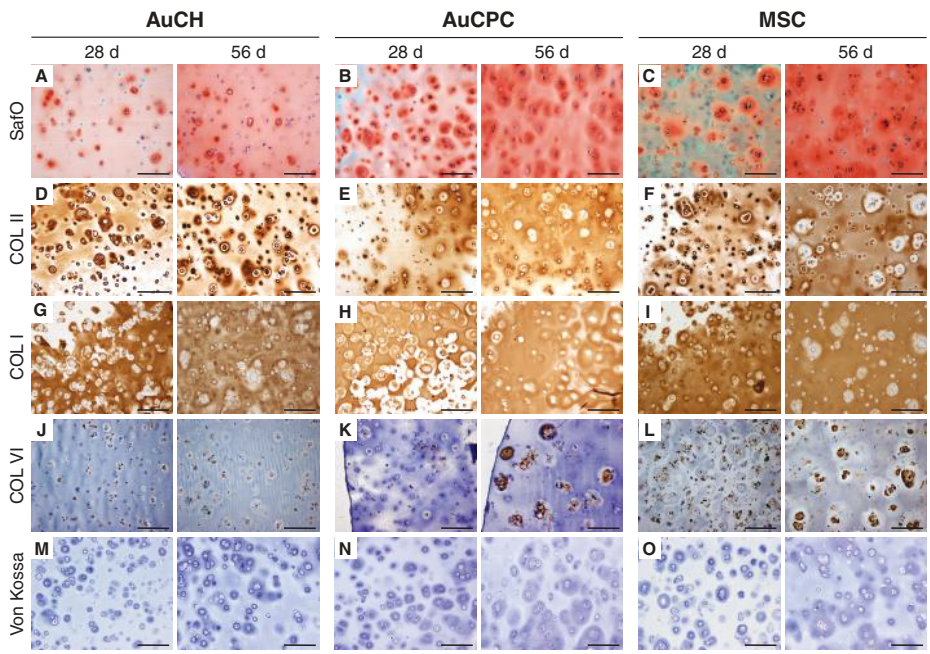

Figure 6. Histological analysis of cell-laden hydrogels after 28 and 56 days in chondrogenic culture confirms cartilage-like matrix deposition. Safranin O staining visualizing proteoglycan deposition in AuCH (A), AuCPC (B) and MSC (C) samples. Immunohistochemistry for collagen type II (D/E/F), collagen type I (G/H/I) and collagen type VI (J/K/L). Von Kossa staining demonstrating the absence of mineralization in all three cell types (M/N/O). Scale bars equal 150 µm.

DISCUSSION

Cell selection for the generation of clinically relevant-size cartilage tissue constructs remains a notable challenge in tissue engineering strategies. Cartilage-derived progenitor cells present

a promising cell source for use in tissue engineering-based clinical therapies, since they can generate large numbers of cells while maintaining chondrogenic differentiation potential [368]. In this study, progenitor cells originating from auricular cartilage demonstrate cartilage formation capacity in a 3D hydrogel system by generating a cartilage-like matrix *in vitro*.

Traditionally, chondrocytes from various cartilage tissues (auricular, articular, costal and nasoseptal) are predominantly used for the engineering of auricular structures. Autologous cells have the greatest clinical potential in view of infectious considerations and adverse immunological response [303]. Nevertheless, the acquisition of sufficient cell numbers remains an important limitation in current tissue engineering approaches, since extensive expansion is required to obtain sufficient numbers of autologous cells for the creation of a large tissue constructs [25]. After only few population doublings *in vitro*, auricular, articular, septal and costal chondrocytes undergo dedifferentiation and lose their capacity for the production of cartilage-specific glycosaminoglycans and collagens [63, 144, 189, 234, 307, 310, 368]. Although some studies have demonstrated cartilage-like tissue formation using extensively expanded chondrocytes, these dedifferentiated cells required the addition of fresh chondrocytes in order to salvage some of the chondrogenic phenotype characteristics [294, 349]. Hence, primary chondrocytes seem less suitable as a single donor source for cartilage tissue engineering of large constructs, yet may continue to be beneficial additions to co-cultures [171, 205, 291].

MSCs have also been successfully applied for the generation of cartilage-like tissue constructs [60, 292, 313, 359]. MSCs are attractive for cartilage tissue engineering as they are harvested from the bone marrow with minimally invasive procedures, can be expanded to yield high cell numbers without losing their tissue-specific phenotype [117], and have multipotent differentiation ability [290]. In 3D culture, MSCs can be directed towards the chondrogenic lineage, and have consequently been applied in numerous studies for the generation of cartilage [60, 292, 313, 359]. In fact, their usage is already being explored in clinical trials such as NCT02037204 (IMPACT), NCT00885729, and NCT01227694 (as registered on clinicaltrials.gov). Nevertheless, the usage of MSCs for cartilage engineering harbors the risk of terminal differentiation of cells and subsequent calcification and ossification of tissues [368]. This results in calcification of the extracellular matrix [120] – a phenomenon that is also observed in the costal cartilage framework implanted during auricular reconstruction surgery – causing an increasingly rigid construct [159]. This is an unfavorable outcome for engineered auricular tissue structures as elasticity is one of the key features of the external ear [256, 272, 373].

Tissue-specific progenitor cells maintain stem cell-like proliferative potential, yet also display tissue-specific phenotypes since they are harvested directly from the target tissue. Environmental influences from their niche *in vivo* prime progenitors to regulated proliferation and differentiation

towards the target tissue, providing a clear advantage over non-tissue-specific stem cells like MSCs. Their proliferative ability addresses the important limitation in cell number acquisition that continues to hamper the translation of large tissue-engineered constructs to clinical application. Cartilage stem/progenitor cells were first identified in the superficial zone of the articular cartilage of the knee [86], yet conclusive definitions on the identity of these cells remain elusive [161]. Nevertheless, it is clear that cartilage tissue harbors a potent subpopulation of cells with distinct abilities from primary chondrocytes and a similar nature to MSCs. The current work presents the first identification and evaluation of this subpopulation of progenitor cells in equine auricular cartilage tissue and their potential for cartilage tissue engineering approaches.

Previous literature reporting progenitor cells from auricular tissue is principally focused on cells originating from the perichondrial layer [179, 180, 346], which is a fibrous connective tissue consisting of fibroblasts and perichondrocytes. Recently, the first demonstration of the presence of an auricular cartilage progenitor population separate from the perichondrium was described by Xue *et al*. (2016) in a porcine species. The proliferation rate of these auricular cartilage stem/progenitor cells (CSPCs) was comparable to bone marrow-derived MSCs. Although the authors reported a higher proliferation rate for perichondrium stem/progenitor cells (PSPCs), chondrogenic differentiation potential was greater for auricular cartilage stem/progenitor cells [377]. PSPCs can differentiate into chondrocytes, yet maintain a fibroblastic morphology [179, 377]. Cells derived from the cartilage tissue appear more apt to differentiate towards the chondrogenic and osteogenic lineages, whereas perichondrium-derived progenitor cells are inclined towards adipogenic differentiation [377]. The cartilage forming potential of cartilage stem/progenitor cells has not yet been explored in 3D biomaterial culture for tissue engineering purposes. Hence, the present study focused on cartilage progenitor cells – derived from the cartilage after complete removal of the perichondrium – for elastic cartilage tissue engineering. Besides fundamental characterization of these putative cartilage progenitor cells, their potential for cartilage regeneration for future therapeutic applications is of major interest.

The results from the characterization of AuCPCs demonstrate similar behavior of these cells to MSCs in terms of multipotency. Like MSCs, AuCPCs have the ability to differentiate towards multiple lineages, as confirmed by positive stainings for bone, adipose tissue and cartilage. Furthermore, AuCPCs, being *CD73*$^{+}$, *CD90*$^{+}$, *CD105*$^{+}$, *CD34*$^{-}$ and *CD45*$^{-}$, displayed a gene expression profile for surface markers that is consistent with the minimal criteria for human MSCs [84]. Additional cell surface marker analysis showed similar gene expression profiles between AuCPCs and bone marrow-derived MSCs. A main difference between AuCHs, AuCPCs and MSCs arose in the expression of *CD13*, which is a marker that is widely expressed by a variety of cell subpopulations, including stem cells [46, 85]. These data in addition to the ability to differentiate towards multiple

lineages demonstrate a behavior similar to bone marrow-derived MSCs, both genetically and functionally.

The limited knowledge on AuCPCs demands further investigation into specific cell characteristics, yet our study focused on advancing towards utilization of these cells. Their stem cell characteristics in addition to a differentiation capacity likely primed towards their source tissue makes AuCPCs a highly interesting cell source for cartilage tissue engineering strategies. These cells provide an opportunity to overcome the drawbacks of the currently used cell types in cartilage tissue engineering and can thereby increase the likelihood of using tissue-engineered auricular cartilage structures for clinical application. Hence, the behavior of AuCPCs in 3D culture for tissue regeneration was explored in comparison to AuCHs and MSCs. Growth and differentiation in the third dimension provides a more natural environment for cells and allows the preservation of tissue-specific characteristics [270]. In this study, cells were encapsulated in a 3D hydrogel system (gelMA) proven to be a permissive environment for neocartilage production [177, 193, 194, 312]. GelMA has become a widespread platform for tissue engineering and bioprinting applications, owing to its natural bioactivity and tailorability [177]. This versatile hydrogel can be functionalized for improved performance in supporting cells (for instance with hyaluronic acid [194]), printing resolution (e.*g.* with gellan gum [228]) or mechanical stability (by incorporating reinforcing fibers [363] or scaffolding materials such as stiff and elastic hydrogels [217] or thermoplastic polymers [226]). Its biocompatibility and clinical grade options make gelMA a beneficial biomaterial choice with respect to future clinical translation [177].

In 3D hydrogel culture, all three cell types demonstrated increasing extracellular matrix production over the culture period, as indicated by sGAG/dsDNA quantification and confirmed by histology. Whereas AuCHs and AuCPCs displayed similar matrix-synthesizing behavior, MSCs outperformed both cell types in terms of sGAG deposition. Proteoglycan aggregates are the major structural matrix components contributing to the mechanical properties of the tissue, and the trend in matrix synthesis clearly matched the compressive moduli among cell types at the end of the culture period. Nevertheless, the observed differences in total sGAG content at 28 days of culture did not reflect the concurrent mechanical properties. At this timepoint, AuCPCs were outperforming MSCs in terms of total sGAG content and compressive Young's modulus, but the marked difference in Young's modulus between AuCPCs and AuCHs was not observed in the total sGAG content. Since the mechanical properties of cartilage tissue are impacted by the organization of the tissue [372], a potential explanation is presented by a more homogeneous distribution of proteoglycans and collagens in both AuCPCs and MSCs upon histological examination, whereas AuCHs displayed a more cluster-like organization of matrix components. In our study, the mechanical properties of tissue-engineered cartilage constructs ranged between 102.8 and 179.2 kPa, which is at least a factor 10 inferior to native auricular cartilage. Griffin *et al.* (2016) reported the compressive moduli

of native human auricular cartilage to range between 1.41 and 2.08 MPa [130], whereas Nimeskern *et al*. (2015) determined an instantaneous modulus of 3.27-11.02 MPa, a maximum stress of 0.87-3.11 MPa, and an equilibrium modulus of 2.22-7.23 MPa [255]. Tissue-engineered cartilage constructs thus require improved mechanical properties, which could be attained through supporting frameworks [54], by fiber reinforcement [363], or possibly through mechanical loading of the developing neo-tissue, which has previously been shown to increase cellular production of matrix components in articular cartilage [233].

Differential mRNA expression of cartilage-specific markers confirmed cell differentiation towards the chondrogenic lineage in all three cell types. Although *COL2A1* expression was highly upregulated over the 56-day culture period, all three cell types demonstrated concurrent *COL1A1* expression, confirmed by immunohistochemistry. Improvement of neocartilage quality may be attained through functionalization of the hydrogel or by optimizing the recipe of the differential culture media. For example, the incorporation of hyaluronic acid in gelMA was shown to decrease collagen type I production [194], whereas supplementation with insulin-like growth factor-1 (IGF-1) was demonstrated to enhance the generation of elastic fibers in addition to improved overall tissue formation [300].

The auricle is a strong yet flexible structure composed of cartilage of the elastic type. Its composition is similar to that of hyaline cartilage, consisting of negatively charged proteoglycan aggregates attracting water and a dense collagen type II network [234]. Nevertheless, the auricular cartilage is unique in that it harbors an intricate network of elastic fibers. Elastin has a defining role in the mechanical properties of elastic cartilage, allowing flexibility and a swift return to its original shape after minor loads [255]. AuCPCs exhibited an upregulated expression of elastin in the cell-laden hydrogels. This feature offers a potential prime advantage of using AuCPCs over MSCs for tissue engineering of auricular cartilage, since the latter did not display elastin upregulation. Quantification and/or visualization of the production of elastin in tissue-engineered constructs could confirm this potential and would be advisable for future studies.

One common problem with cartilage tissue engineering is calcification of the neo-tissue [159]. Primary chondrocytes may terminally differentiate and become hypertrophic, which can lead to calcification and eventually ossification of the neo-tissue [121, 288]. Collagen type X is a typical marker of chondrocyte hypertrophy [213], although its presence has been demonstrated in native non-mineralized auricular cartilage [76, 142]. Several studies report the expression of collagen type X in cartilage tissue engineered from auricular chondrocytes, yet this did not result in mineralization of neotissues in both *in vitro* and *in vivo* conditions [76, 142]. Indeed, in this study, the mRNA expression of *COL10A1* was highly upregulated in AuCHs, indicating chondrocyte hypertrophy. Nonetheless, our findings confirm that this did not result in mineralization of the

neotissue up to 56 days of *in vitro* culture. The relatively low expression of *COL10A1* in both AuCPCs and MSCs may indicate preservation of phenotype in these cell types under *in vitro* chondrogenic conditions. Nevertheless, the expression of *RUNX2* was significantly upregulated in MSCs compared to AuCPCs and AuCHs at 56 days of culture. *RUNX2* drives osteogenic differentiation and inhibits differentiation of MSCs into chondrocytes [182]. In contrast, AuCPCs demonstrated a significant reduction in *RUNX2* expression over time. MSCs from the bone marrow have been shown to form bone *in vivo* through the endochondral ossification pathway [120], whereas AuCPCs originate from the cartilage itself and their niche may have primed them towards the target tissue, maintaining their specific phenotype. The lack of hypertrophy in AuCPC cultures, as well as the decreased *RUNX2* and increased elastin expression levels point to a stable phenotype that is amenable for tissue engineering.

CONCLUSION

The identification of a cartilage progenitor subpopulation in the auricular cartilage provides access to a promising cell source for tissue engineering strategies for auricular reconstruction. Although under the current culturing conditions, bone marrow-derived MSCs seem to perform better in terms of matrix production, major advantages of AuCPCs include the ability to generate high cell numbers [368, 377], upregulation of the elastin gene, and a limited endochondral ossification potential. These advantages taken together make progenitor cells from the auricular cartilage a highly interesting candidate as a cell source in future tissue engineering-based clinical therapies.

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SUPPLEMENTARY INFORMATION

Supplementary Table 1. Primer sequences applied for the characterization of AuCH, AuCPC and MSC. RT-PCR forward (Fw) and reverse (Rv) primer sequences for several genes as well as the housekeeping gene hypoxanthine phosphoribotransferase 1 (*HPRT1*). Expected amplicon sizes are expressed in base pairs (bp).

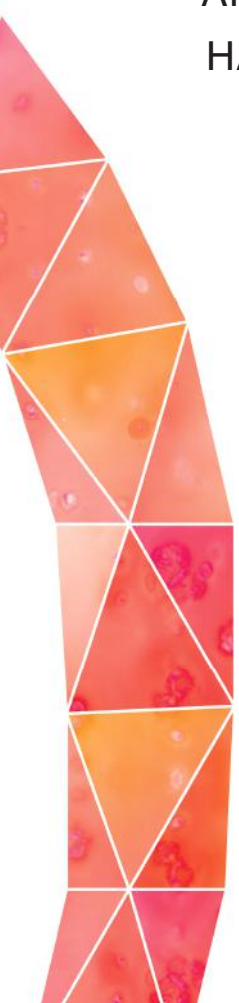
Target gene	Primer sequence (5′ to 3′)	Amplicon size (bp)
HPRT1	Fw: CAAGCTTGCTGGTGAAAAG	95
	Rv: GGCATATCCTACGACAAACT	
CD13	Fw: CTGAGTGGAGAGACAGAGTA	147
	Rv: CTGGAAATACTCGAAGAGGG	
CD29	Fw: CTGGAGATGGGAAACTTGG	229
	Rv: GTTCCTACTGCTGACTTAGG	
CD31	Fw: CAGAATCCTTCTCTATGCCC	194
	Rv: CATGGCCATCACTGAGTAG	
CD34	Fw: GACTCAAGGTATCTGCCTG	104
	Rv: CCTGTTCTTTCTCACAGAGG	
CD44	Fw: CTGGGGACTCTGCCTC	99
	Rv: TAGCGGCCATTTTTCTCC	
CD45	Fw: TTGAACGGCCTTGAACC	153
	Rv: CTTGGCACCTTCAGTACC	
CD49d	Fw: CTACAACTTGGACACCGAG	201
	Rv: GTCCGGTCTGGATTCTTTC	
CD73	Fw: TCCGGACTTTATTTGCCG	346
	Rv: CAGAGGTGACTATGAATGGG	
CD90	Fw: CTCTACACATGCGAACTCC	90
	Rv: CTCGCACTTGACCAGTTT	
CD105	Fw: CAGTAATGAGGTGGTCGTC	108
	Rv: CTGAGGTAGAGGCCCAG	
CD106	Fw: ACTCTTACTTGTGCACGG	103
	Rv: CCACTGAAACTGATCTCTGG	
CD146	Fw: TCCGTGTGTACAAAGCTC	137
	Rv: GTACCAGATGACCTGAGGA	
CD166	Fw: GTCTTCTGCCTCTTGATCG	223
	Rv: CTGTCTTTGTACTCTGGGAC	

Supplementary Table 2. Primer sequences applied for the evaluation of cartilage-like tissue synthesis. qPCR forward (Fw) and reverse (Rv) primer sequences for several genes indicating cartilage or bone synthesis, as well as the housekeeping gene hypoxanthine phosphoribotransferase 1 (*HPRT1*). Expected amplicon sizes are expressed in base pairs (bp).

Target gene		Primer sequence (5′ to 3′)	Amplicon size (bp)
HPRT1	Hypoxanthine phosphoribotransferase 1	Fw: AAGCTTGCTGGTGAAAAG	95
		Rv: GCATATCCTACGACAAACT	
ACAN	Aggrecan	Fw: AAGACAGGGTCTCGCTGCCCAA	115
		Rv: ATGCCGTGCATCACCTCGCA	
COL1A1	Collagen type I, α1 chain	Fw: CGTGACCTCAAGATGTGC	94
		Rv: AGAAGACCTTGATGGCGT	
COL2A1	Collagen type II, α1 chain	Fw: GGCAATAGCAGGTTCACGTACA	79
		Rv: CGATAACAGTCTTGCCCCACTT	
COL10A1	Collagen type X, α1 chain	Fw: GGGAAACGGGATATGGTGCT	168
		Rv: GTCCCCTTTCTCCCGGAATG	
COMP	Cartilage oligomeric protein	Fw: CCACGTGAATACGGTCACAG	104
		Rv: ACGTCTGCTCCATCTGCTTC	
RUNX2	Runt-related transcription factor 2	Fw: GCAAGGTTCAACGATCTGA	248
		Rv: GGGACACCTACTCTCATACT	
ELASTIN	Elastin	Fw: TGGAGTCCCAGGTGTTGTTG	137
		Rv: CATAGCCAGGAACCCCGAA	

CHAPTER 4

FIBRONECTIN-ADHERING PROGENITOR CELLS RESIDE IN HUMAN ADULT, PEDIATRIC AND MICROTIA AURICULAR CARTILAGE AND HARBOR A POTENTIAL FOR REGENERATIVE EAR RECONSTRUCTION



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ABSTRACT

Tissue engineered auricular cartilage constructs can open new avenues to overcome donor site morbidity and unsatisfactory aesthetic outcomes related to the current state-of-the-art treatment of microtia. Remaining challenges include the acquiring sufficient regeneration-competent cells and the subsequent production of high quality neocartilage. Auricular cartilage progenitor cells (AuCPC) are a resident subpopulation of the native tissue and harbor a high proliferative potential as well as cartilage-forming capacity. The purpose of this study was to identify progenitor cells in the human auricular cartilage – either healthy or affected by congenital deformities, and from donors having different age profiles – and to assess their potential for cartilage regeneration. Human auricular cartilage progenitor cells were isolated from adult, pediatric and microtia cartilage. The proliferative and multipotent qualities of these cells were characterized through growth rate determination, trilineage differentiation and flow cytometry. Subsequently, AuCPCs were encapsulated in a 3D hydrogel and cultured *in vitro* for 56 days, during which biochemical, mechanical and histological assessment was performed to evaluate chondrogenesis. The presence of fibronectin-adhering, colony-forming cells was confirmed in human adult and pediatric cartilage as well as the rudimentary microtia cartilage. These cells could differentiate towards bone, adipose tissue and cartilage over multiple passages. High expression rates of *CD90*, *CD105* and *CD73* were observed. AuCPCs exhibited growth rates between 0.43 and 1.49 population doublings per 24 hours, varying between passages. Upon chondrogenic differentiation, cell-laden hydrogels demonstrated upregulated cartilage-specific markers like aggrecan, collagen type II and cartilage oligomeric matrix protein over time, as well as low expression levels of collagen type X and runt-related transcription factor 2. Glycosaminoglycan content increased significantly over the culture period, as confirmed by histology. Immunohistochemical analysis showed positive staining for collagen type II, collagen type I and elastin. We describe, for the first time, the presence of fibronectin-adhering cartilage progenitor cells in the human auricular cartilage, including the rudimentary microtia cartilage. These cells demonstrate a potent ability to proliferate without losing their multipotent differentiation ability, and to produce cartilage-like matrix in a 3D culture. As these novel cells can be easily obtained through a non-deforming biopsy of the healthy ear or from the otherwise redundant microtia cartilage, they may provide an important solution to long-existing challenges in auricular cartilage tissue engineering.

BACKGROUND

Microtia is a developmental disorder of the external ear, which results in a range of auricular deformities spanning from minimal structural anomalies to a complete absence of the auricle. Worldwide, 0.8 – 4.2 per 10.000 children are born with this usually unilateral condition [3]. Although relatively uncommon, having this visible deformity is burdensome for both children and adults. The unusual appearance of the auricle often causes teasing and a reduced self-confidence, impacting social life, career, and leisure activities. Anxiety, depression, and behavioral problems are also reported in microtia patients [146]. Psychosocial functioning improves significantly after surgical correction of the affected ear [146, 162, 321].

The current golden standard in the treatment of microtia is auricular reconstruction surgery using autologous cartilage tissue. In this procedure, cartilage grafts are taken from the patient's ribs and skillfully carved into a framework that mimics the contours of the contralateral normal ear [17]. Although decent aesthetic results can be obtained with this approach, there are important drawbacks. Firstly, as the ear is as unique as a fingerprint [147], auricular reconstruction is perceived as one of the most challenging procedures in plastic surgery [207]. Even in experienced hands, the results from reconstructive surgery are not always consistent [17]. Secondly, the carved framework is considerably different from the delicate three-dimensional structure of the native auricle in terms of fine anatomy and mechanical properties: the reconstructed fibrocartilage framework is slightly thicker and less flexible in comparison to the native elastic cartilage. In addition, symmetrical projection from the skull is difficult to achieve [25, 159]. As the costal cartilage is prone to calcification, over time the definition of the carved frame can become less pronounced and more rigid [159]. Thirdly, there is a risk of post-operative infection at both operative sites or necrosis of the skin overlying the cartilage frame. Lastly, harvesting a large chunk of cartilage from the ribs can cause a visible chest deformity, a wide scar on the chest, and has a risk of complications including pneumothorax [65, 159]. Synthetic implants such as those made of silicone or porous polyethylene eliminate donor site morbidity and framework problems from the equation, yet they are still deemed less favorable due to risk of implant fracture and occurrences of extrusion through the skin after infection or light traumas [15, 17, 53].

Tissue-engineered implants can open new avenues to overcome the aforementioned donor site morbidity and unsatisfactory aesthetic outcomes related to the current treatment. Tissue engineering technologies allow for the creation of new cartilage *in vitro* by using a combination of cells, bioactive cues and supporting materials to grow new tissue [184, 187]. Using these principles as a therapeutic approach would obviate harvesting and sculpting the costal cartilage framework, consequently decreasing operating time and avoiding donor site morbidity. Despite great advances in cartilage tissue engineering, two main challenges in engineering the elastic

cartilage of the human auricle remain. Firstly, a significant number of cells is required for the generation of a cartilage construct the size of the human auricle: estimates range between 100 and 250 million cells [25, 66]. Secondly, the quality of engineered cartilage is still suboptimal with regards to structure, component ratios, biocompatibility, functionality and durability [25, 243, 266, 324]. Specifically, the neo-tissue often exhibits fibrous characteristics or calcifications [26, 159, 167, 185, 305]. In addition, a critical characteristic of the external ear is its flexibility, allowing the auricle to bend without breaking. This flexibility is achieved through the presence of elastic fibers in the tissue, which is accordingly classified as elastic cartilage [25, 130, 257, 272, 302, 390]. Hence, the production of elastic fibers in engineered cartilage tissue will greatly contribute to the construct's flexibility. The overall success of a tissue-engineered auricular cartilage implant is largely determined by the quality of the produced tissue. Consequently, choosing an appropriate cell type is crucial in overcoming the hurdles of quantity and quality.

Cell-based tissue engineering of the human auricle thus requires a high cell yield and the ability of the chosen cell type to produce cartilage-specific extracellular matrix to recapitulate the biochemical and mechanical properties of the native elastic auricular cartilage. Options include primary chondrocytes, mesenchymal stromal cells (MSC) and more recently also cartilage progenitor cells (CPC). Chondrocytes naturally possess a chondrogenic determination, yet they rapidly lose their phenotype upon expansion *in vitro* [144, 288, 303, 307]. As such, the use of this cell type would require a very large donor site in order to obtain a sufficient number of cells to create the human auricle. Mesenchymal stem cells, in contrast, have a high expansion capacity *in vitro* [117] but exhibit a tendency to undergo hypertrophic differentiation upon long-term *in vitro* and *in vivo* culture, which can result in the formation of calcified cartilage. This template can then be remodeled into bone through the process of endochondral ossification, leading to undesirable tissue calcifications contributing to implant stiffness [120, 229]. Despite numerous strategies, including redifferentiation of chondrocytes [211, 294, 338, 349, 355] or co-culturing MSCs with chondrocytes [66, 171, 200, 291, 382], translation of tissue-engineered auricular cartilage towards clinical application remains hampered by the requisite of sufficient cell quantities able to produce adequate quality neocartilage.

Cartilage progenitor cells originate in the native cartilage tissue and have been shown to exhibit a high proliferative capacity and to retain multipotency upon expansion [368, 377]. CPCs isolated from equine auricular and articular cartilage have been shown to produce cartilage-like tissue in an *in vitro* 3D hydrogel model [193, 265]. In addition, auricular CPCs were shown to exhibit a significant reduction of the expression of *RUNX2* – the master transcription factor for hypertrophy and osteogenesis [265]. These cartilage progenitor cells can thus potentially overcome the aforementioned problems encountered with the use of chondrocytes and MSCs in auricular cartilage tissue engineering.

The purpose of this study was to identify progenitor cells in the human auricular cartilage and to assess their potential for cartilage regeneration. We describe the presence of auricular cartilage progenitor cells (AuCPC) in human auricular cartilage from different donor sources. The proliferative and multipotent qualities of progenitors sourced from adult, pediatric and rudimentary microtia auricular cartilage were characterized throughout multiple passages. In addition, cells were encapsulated in a 3D hydrogel system and cultured in chondrogenic differentiation medium for a period of 56 days during which biochemical, mechanical and histological assessment was performed to evaluate the chondrogenic capacity of these cells for use in tissue engineering strategies.

MATERIALS & METHODS

Harvest of human auricular cartilage

For the isolation of human auricular cartilage progenitor cells (AuCPC), fresh auricular cartilage was collected from three sources: recently deceased elderly donors (AuCPC-adult; n = 4, mean age 87.5 ± 12.3, range 69-94 years), healthy normal cartilage of pediatric patients removed during protruding ear correction surgery (AuCPC-pediatric; n = 3, mean age 7.7 ± 2.1, range 6-10 years), and the cartilage remnants of pediatric patients with microtia, removed during ear reconstruction surgery (AuCPC-microtia; n = 3, mean age 10 ± 3.6, range 7-14 years). Tissues were kindly provided by the Department of Anatomy at the University Medical Center Utrecht (The Netherlands) and the Department of Plastic, Reconstructive & Hand Surgery at the Wilhelmina Children's Hospital (Utrecht, The Netherlands). All tissues were obtained from biopsies of redundant tissue excised during surgery or from deceased donors who had donated their body to science, according to the guidelines of the Ethical Committee of the University Medical Center Utrecht. Anonymization of donated tissue was performed to ensure non-traceability of their origins.

Isolation of human chondroprogenitor cells

Harvested auricles from deceased donors were thoroughly washed with water and soap and subsequently disinfected by soaking in Betadine® (Meda Pharma, The Netherlands). Under sterile conditions, the auricular skin and subcutaneous tissue were removed using a scalpel. Microtia and protruding ear cartilage remnants were washed in phosphate-buffered saline (PBS) and subsequently stripped of any remaining subcutaneous tissue. In all cases, the perichondrial layer was removed using a scraping technique as previously described [265]. Cartilage chips were sectioned off the exposed cartilage layer, washed in PBS substituted with 0.3 % gentamycin (Lonza, USA) and minced into 1 mm^2 pieces. The minced cartilage tissue was enzymatically digested in 0.2 % pronase (Roche, USA) solution for 2 hours followed by 0.075 % collagenase type II (Worthington Chemical Corporation, USA) digestion for 16 hours at 37 °C. The solution was then filtered through a 100 mm cell strainer and centrifuged for 5 minutes at 300 ×g to obtain a cell pellet. The pelleted cells

were resuspended in Dulbecco's modified Eagle medium (DMEM; 31966, Gibco, The Netherlands) and subjected to a fibronectin adhesion assay as previously described [86, 368]. Briefly, cells were plated at a density of 500 cells/cm^2 in fibronectin-coated culture flasks and incubated for 20 minutes at 37 °C. The non-adherent cells were carefully removed and the remaining attached cells were cultured in chondroprogenitor expansion media, consisting of DMEM supplemented with 10 % v/v fetal bovine serum (FBS; Lonza), 0.2 mM L-ascorbic acid 2-phosphate (Sigma-Aldrich, The Netherlands), 100 U/ml penicillin (Life Technologies, The Netherlands), 100 µg/mL streptomycin (Life Technologies) and 5 ng/mL basic fibroblast growth factor (bFGF; Peprotech, UK). Cells were collected and stored at each passage up till passage 4 in liquid nitrogen until further use.

Visualization of cell morphology during expansion

Morphological evaluation of AuCPC was carried out from passage 0 through 5 by light microscopy imaging (Leica DMi1, Germany). Colony formation was captured during passage 0. At subsequent passages, images were taken at day 4 of culture.

Evaluation of growth rates during expansion

Proliferation rates were determined at passages 1-5 using a resazurin assay [74]. AuCPC cells from all donors were cultured up to confluency at every passage and subsequently plated at a density of 5.0 × 10^3 in 12-well tissue plates (4 replicates per donor), where they were cultured in progenitor expansion medium supplemented with 5 ng/mL bFGF. On days 1, 3, 4-10 (or beyond if cell numbers had not reached plateau growth phase) the assay was performed by incubating the cells in 10x diluted resazurin solution (Alfa Aesar, Germany) for 3 hours at 37 °C. Fluorescence of resorufin, the metabolically reduced compound, was measured at 544 nm excitation and 570 nm emission using a spectrofluorometer (Fluoroskan Ascent FL; ThermoFisher, USA). A calibration curve was determined by plating known cell densities and measuring the absorbance at day 1. Population doublings were calculated using the following equation, where $x0$ is the starting cell number and N is the cell number at time of measurement:

$$\text{Population doublings} = \frac{\log\left(\frac{N}{x0}\right)}{\log 2}$$

Determination of stem cell marker expression using flow cytometry analysis

Flow cytometry was used to determine stem cell marker expression of the isolated cell population of each donor, using a marker panel consisting of *CD45*, *CD34*, *CD73*, *CD90* and *CD105* [84]. For each donor, 1.0 × 10^5 AuCPCs at passage 4 were washed in 1X Flow Cytometry Staining Buffer (R&D Systems, USA) and incubated for 45 minutes at room temperature in the dark with either CD90-APC (R&D Systems), CD105-APC (Abcam, UK), CD73-CFS (R&D Systems) or a cocktail of

negative markers conjugated to PE (consisting of CD45-PE Mouse IgG_1 Clone 2D1, CD34-PE Mouse IgG_1 Clone QBEnd10, CD11b-PE Mouse IgG_{2B} Clone 238446, CD79A-PE Mouse IgG_1 Clone 706931, HLA-DR-PE Mouse IgG_1 Clone L203; R&D Systems). Labeled cells were washed once with and subsequently resuspended in 100 µL Staining Buffer, and analyzed using a BD FACSCanto II (BD Biosciences, USA). Dead cells were excluded with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Corresponding isotype antibodies were used as controls to exclude non-specific binding. Results were analyzed using FlowJo V10 data analysis software package (TreeStar, USA).

Assessment of multipotency through trilineage differentiation

Retention of multipotency of hAuCPCs during expansion was evaluated through an *in vitro* trilineage differentiation assay at passages 3, 4 and 5. Cells were directed towards the osteogenic, adipogenic or chondrogenic lineage through the appropriate differentiation media. For adipogenic and osteogenic differentiation, cells were plated in duplicate at a density of 3×10^5 cells per well in 6-well tissue culture plates and cultured until sub-confluency in chondroprogenitor expansion medium. When cell-cell contact was observed, cells were cultured in differentiation media for 21 and 28 days, respectively. Osteogenic differentiation medium consisted of αMEM (Gibco) supplemented with 10 % v/v FBS (Lonza), 100 U/mL penicillin (Life Technologies), 100 µg/mL streptomycin (Life Technologies), 0.2 mM L-ascorbic acid 2-phosphate (Sigma-Aldrich), 20 mM β-glycerol phosphate (Sigma-Aldrich) and 100 nM dexamethasone (Sigma-Aldrich). Adipogenic medium consisted of αMEM (Gibco) supplemented with 10 % v/v FBS (Lonza), 100 U/mL penicillin (Life Technologies), 100 µg/mL streptomycin (Life Technologies), 0.01 mM indomethacin (Sigma-Aldrich), 83 mM 3-Isobutyl-1-metylxanthine (Sigma-Aldrich) and 1.72 µm bovine pancreas-derived insulin (Sigma-Aldrich). For chondrogenic differentiation, cells were pelleted at a density of 2.5×10^5 in 15 mL Falcon® tubes by centrifugation at 300 ×*g* for 5 minutes. The pellets were subsequently cultured for 21 days in chondrogenic differentiation medium, consisting of DMEM supplemented with 1 % v/v ITS+ Premix (insulin-transferrin-selenous acid; Corning, USA), 0.2 mM L-ascorbic acid 2-phosphate (Sigma-Aldrich), 100 U/mL penicillin (Life Technologies), 100 µg/ mL streptomycin (Life Technologies), 100 nM dexamethasone (Sigma-Aldrich) and 10 ng/mL transforming growth factor β1 (TGF-β1; Peprotech). Culture medium was refreshed every 3 days.

At the end of the culture period, cells and pellets were washed with PBS and fixed in 4 % neutral buffered formalin (NBF; Klinipath, UK). Pellets were subsequently embedded in paraffin and sectioned into 5 µm-thick slices. Osteogenic differentiation was determined by observing calcified matrix deposition using Alizarin Red S staining (Sigma-Aldrich). Adipogenic commitment was visualized by Oil Red O staining (Sigma-Aldrich) demonstrating the formation of intracellular lipid vesicles. Chondrogenic differentiation was assessed by staining sectioned pellets with Safranin O (Sigma-Aldrich) to visualize glycosaminoglycan deposition.

Fabrication of cell-laden hydrogel constructs for 3D culture

Gelatin methacryloyl (gelMA) was synthesized according to a previously published protocol, and used as a platform to produce hydrogels for 3D tissue culture [218]. Briefly, gelatin type A (obtained from porcine skin; Sigma-Aldrich) in PBS was functionalized with methacrylic anhydride groups to achieve an 80 % degree of functionalization of the available primary amines. Subsequently, a 10 % w/v solution of gelMA was supplemented with 0.1 % w/v 2-hydroxy-1-[4-(2-hydroxyethoxy) phenyl]-2-methyl-1-propanone (Irgacure 2959; BASF, Germany) as a photoinitiator. AuCPCs of each donor were expanded to passage 4 and were encapsulated in the hydrogel at a density of 1.5×10^7 cells/mL at 37 °C. The cell-laden gel was cast into a custom-made Teflon™ mold and subsequently subjected to UV-radiation for 15 minutes (wavelength $\lambda = 365$ nm, intensity $E = 7$ mW/cm^2, at height of 12 cm; CL-1000L UV Crosslinker, UVP, UK) to allow free-radical polymerization crosslinking of the hydrogel, producing cylindrical samples (diameter = 6 mm, height = 2 mm). As controls, cell-free hydrogel samples were prepared under the same conditions. All samples were cultured in chondrogenic differentiation medium for 1, 28 and 56 days at 37 °C and 5 % CO_2 and receiving fresh media 3 times per week.

Gene expression of cartilage markers after chondrogenic differentiation

After 1 and 56 days of culture, the relative gene expression of cartilage markers in cell-laden hydrogels (3 replicates per donor per timepoint) was evaluated through qPCR. Analyzed markers included aggrecan (*ACAN*), cartilage oligomeric matrix protein (*COMP*), collagen type I (*COL1A1*), collagen type II (*COL2A1*), collagen type X (*COLXA1*), and runt-related transcription factor 2 (*RUNX2*). Expression levels of these markers were normalized against the housekeeping gene hypoxanthine phosphoribosyltransferase (*HPRT1*). Primer sequences for each transcript are reported in Supplementary Table 1. At each given timepoint, cell-laden hydrogel samples were mechanically ground in RLT buffer (Qiagen, Germany) and mRNA was isolated from the lysate using the RNeasy Mini Kit (Qiagen) and subsequently quantified with a Nanodrop 2000 (Thermo Scientific, The Netherlands). A SuperScript® III Platinum SYBR Green One-Step qRT-PCR Kit (Life Technologies) was used for mRNA amplification and cDNA synthesis, which was performed with a LightCycler® 96 (Roche). Relative gene expression, Ct and efficiency values were calculated using the PCRminer algorithm [386].

Biochemical analysis of cell-laden hydrogels

After 1, 28 and 56 days of culture, 4-6 replicates of each group of cell-laden hydrogels were collected for quantification of DNA and GAG content. Samples were frozen at -20°C and subsequently lyophilized. The wet and dry weights were recorded during this process to calculate the final mass of the lyophilized samples. Subsequently, samples were digested overnight at 60 °C in 200 µL papain digestion buffer (P3125; Sigma-Aldrich), consisting of 0.2 M NaH_2PO_4 (Merck, USA) and 0.01 M ethylenediaminetetraacetic acid (EDTA; VWR, USA) in milliQ water (pH = 6.0),

supplemented with 250 µL/mL papain solution (48 units/mg of protein; Sigma-Aldrich) and 0.01 M cysteine (C9768; Sigma-Aldrich).

Total double-stranded DNA (dsDNA) content was quantified using a Quant-iT PicoGreen dsDNA assay (Life Technologies). Fluorescence was measured at 485 nm excitation and 520 nm emission with a spectrofluorometer (Fluoroskan Ascent FL; ThermoFisher). Results were corrected for the dilution factor and compared to a standard of known concentrations of DNA.

Glycosaminoglycan content, as a measure of cartilage-specific matrix production, was quantified using a demethylmethyleneblue (DMMB; Sigma-Aldrich; pH = 3.0) assay. The 525/595 nm absorbance ratio of the reagent was measured with a VersaMax plate reader (Molecular Devices, UK). The content of sulphated GAG (sGAG) was derived using a standard of known concentrations of chondroitin sulphate C and corrected for the dilution factor.

Total sGAG and dsDNA content in each sample were both normalized against the dry weight of the sample. The ratio of sGAG per dsDNA was calculated to display the cartilage-specific matrix-production activity of single cells in the hydrogel.

Compressive mechanical testing of cultured hydrogel constructs

After 1, 28 and 56 days of culture, 4-6 replicates per timepoint were collected for each donor and subjected to an unconfined uniaxial compression test to evaluate the mechanical properties. Using a dynamic mechanical analyzer (DMA Q800; TA Instruments, Belgium), samples were compressed at a -20 %/min strain rate to a maximum of -30 % strain. The Young's modulus of each sample was calculated as the slope of the initials linear segment (10-15 % strain) of the stress-strain curve.

Visualization of matrix production through histology & immunohistochemistry

Deposition of key components of cartilage extracellular matrix in cell-laden hydrogels after 1, 28 and 56 days of culture was visualized by histology and immunohistochemistry on formalin-fixed, paraffin-embedded samples. After fixation in 4 % neutral buffered formalin, samples were dehydrated through a graded ethanol series (70 %, 96 % and 100 % ethanol), cleared in xylene and embedded in paraffin. Samples were sectioned into 5 µm-thick slices and deparaffinized prior to staining. A triple stain consisting of Weigert's hematoxylin (cell nuclei), Fast Green (collagens) and Safranin O (proteoglycans) was performed to visualize cartilage glycosaminoglycan deposition. Deposition of collagens was evaluated by immunohistochemistry, with appropriate antibodies for collagen type I (ab138492, 1:400; Abcam) and collagen type II (II-II6B3; DSHB, Iowa, USA). Appropriate IgG were used as isotype controls. After deparaffinization, samples were first treated with 0.3 % v/v H_2O_2 to block endogenous peroxidases. Antigen retrieval was performed with 1 mg/mL pronase (Roche) and 10 mg/mL hyaluronidase (H2126; Sigma-Aldrich), both applied for

30 minutes at 37 °C. Subsequently, the tissue sections were blocked with bovine serum albumin (BSA; 5 % w/v in PBS) for 1 hour at room temperature. Then, the primary antibodies were incubated overnight at 4 °C, after which an HRP-tagged secondary antibody was applied for 1 hour at room temperature. For the collagen type II staining, Goat Anti-Mouse HRP (p0447, 1:200; DAKO) was used, and for the collagen type I staining HRP-conjugated EnVision+ for Rabbit (K4010; DAKO) was used. The staining was developed with 3,3-diaminobenzidine-horseradish peroxidase (Sigma-Aldrich) and cell nuclei were counterstained with Mayer's hematoxylin. The formation of elastin was also evaluated by immunohistochemistry. After deparaffinization and blocking, antigen retrieval was performed with 0.25 % trypsin in EDTA (25200; Gibco) applied for 30 minutes at 37 °C. Then, tissue sections were blocked with BSA for 30 minutes at room temperature. The primary antibody (ab9519, 1:20; Abcam), Biotinylated Anti-Mouse IgG (RPN1001V, 1:200; GE Healthcare, The Netherlands), and streptavidin conjugated with HRP (P0397, 1:500; DAKO) were subsequently applied, each for 1 hour at room temperature, with washing in between. The staining was developed with 3,3-diaminobenzidine-horseradish peroxidase and cell nuclei were counterstained with Mayer's hematoxylin. All sections were mounted in DPX mounting media (Millipore, USA) and imaged using a light microscope (Olympus BX51; Olympus, Germany).

Statistical analysis

Quantitative results are expressed as mean ± standard error of the mean (SEM). Quantitative analyses were performed through two-way ANOVA with a Bonferroni post-hoc test. Statistical analyses were carried out using Graphpad Prism 7 (Graphpad Software, USA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Human auricular cartilage progenitor cells demonstrate stem cell potency

Progenitor cells isolated from the auricular cartilage of adult, pediatric and microtia sources all exhibited the ability for plastic adherence and colony formation (Figure 1A-C). Cells from all donors manifested a fibroblast-like morphology with a polygonal and spindle-shaped appearance, which did not change over several passages until passage 5 (Supplementary Figure S1).

Proliferation rates in each donor group varied per passage. In adult AuCPCs, population doublings per 24 hours increased from 0.77 ± 0.07 at passage 1 to 1.49 ± 0.12 at passage 3, after which the rate decreased to 0.58 ± 0.05 doublings at passage 5 (Figure 1D). Pediatric AuCPCs showed a similar trend, starting at 0.43 ± 0.03 doublings per 24 hours at passage 1 and peaking at passage 2 with 1.15 ± 0.15 doublings, after which values marginally decreased (Figure 1E). Microtia AuCPCs demonstrated a proliferation rate of 0.49 ± 0.06 doublings per 24 hours at passage 1, increasing up to a peak value of 1.24 ± 0.20 during passage 4 (Figure 1F).

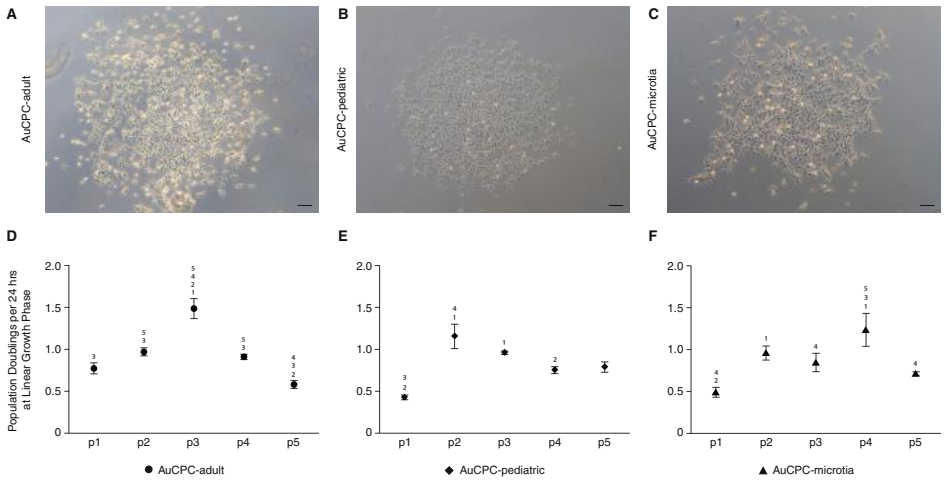

Figure 1. Colony formation capacity and proliferation rates. Isolated progenitor cells sourced from adult (A), pediatric (B) and microtia (C) cartilage demonstrated the ability to form colonies at passage 0. Scale bars equal 100 µm. Proliferation rates were determined at passages 1-5 and are presented as population doublings per 24 hours for adult (D), pediatric (E) and microtia (F) progenitors. Statistically significant differences of $p < 0.05$ are indicated by numbers that refer to the compared passage number, *e.g.* [1] represents a significant difference to passage 1 (p1).

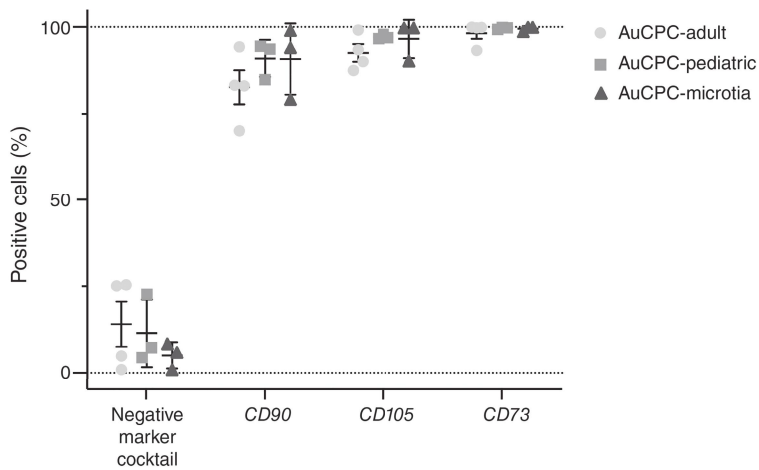

Figure 2. Expression of putative stem cell markers. Using flow cytometry, expression of mesenchymal stromal cell specific markers was measured. High percentages of cells positive for *CD90*, *CD105* and *CD73* were found in adult, pediatric and microtia populations at passage 4. All populations exhibited a low percentage of expression of a panel of surface markers. This negative marker cocktail consisted of *CD11b*, *CD34*, *CD45*, *CD79a*, and *HLA-DR*.

Flow cytometry determined the expression of markers typically used to characterize mesenchymal stromal cells [84] in each donor (Figure 2). Of adult AuCPCs, 82.73 ± 4.93 % expressed *CD90*, 92.58 ± 2.53 % expressed *CD105* and 98.25 ± 1.65 % expressed *CD73*. In pediatric AuCPCs, *CD90* was expressed in 91.03 ± 3.08 %, *CD105* in 97.20 ± 0.36 % and *CD73* in 99.70 ± 0.15 %. Microtia AuCPCs expressed *CD90* in 90.83 ± 5.94 %, *CD105* in 96.63 ± 3.17 % and *CD73* in 99.57 ± 0.43 %. Histograms for each group and each marker are presented in Supplementary Figure S2.

Trilineage differentiation assays confirmed that AuCPC-adult, AuCPC-pediatric and AuCPC-microtia exhibited multipotency potential over several passages (passage 4 is shown in in Figure 3, and passages 3 and 5 are shown in Supplementary Figure S3 and S4 respectively). Upon stimulation with the appropriate culture media, an abundant presence of calcifications (Figure 3A-C), adipose vesicles (Figure 3D-F) and glycosaminoglycans (Figure 3G-I) was observed, indicating successful differentiation into the osteogenic, adipogenic and chondrogenic lineages respectively.

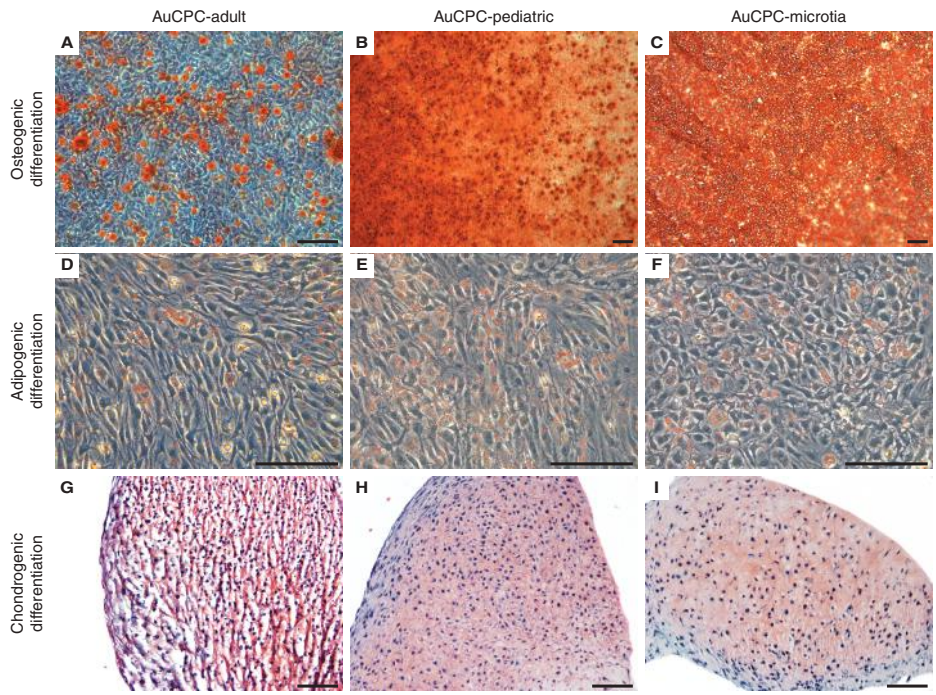

Figure 3. AuCPCs demonstrated a trilineage differentiation capacity in passage 4. AuCPCs sourced from adult, pediatric and microtia cartilage demonstrated the ability to differentiate towards the osteogenic, adipogenic and chondrogenic lineages. Upon stimulation with osteogenic culture media, AuCPCs produced mineralizations (A/B/C). Abundant lipid vesicles were observed in adipogenic culture (D/E/F). Pelleted cells in chondrogenic differentiation media demonstrated the deposition of glycosaminoglycans (G/H/I). Scale bars equal 100 µm.

Differential mRNA expression in hydrogel culture shows chondrogenic marker profile expression

Upon embedding in 3D hydrogel constructs, AuCPCs demonstrated an upregulation of cartilage-specific genes (*ACAN*, *COL2A1* and *COMP*) after chondrogenic culture. In addition, low expression levels of markers indicating chondrocyte hypertrophy (*COL10A1*) and osteogenic differentiation (*RUNX2*) were observed.

Compared to the housekeeping gene *HPRT1*, aggrecan expression (Figure 4A) increased non-significantly from a 2.99-fold (± 0.78) increment in adult AuCPCs, 12.80-fold (± 8.89) in pediatric AuCPCs and 7.81-fold (± 4.96) in microtia AuCPCs at day 1 to 27.52-fold (± 4.36), 29.09-fold (± 18.76) and 53.93-fold (± 44.49) at day 56. Similarly, *COL2A1* expression (Figure 4B) increased non-significantly from 0.02 (± 0.02), 2.06 (± 2.17) and 0.19 (± 0.30) at day 1 to 19.27 (± 3.64), 28.77 (± 26.21) and 57.11 (± 35.40) at day 56 in adult, pediatric and microtia AuCPCs respectively. The expression of *COMP* (Figure 4C) also increased over time in all groups. A significant increment was observed in adult AuCPCs, rising from a 0.09-fold reduction (± 0.03) at day 1 to a 6.59-fold upregulation (± 1.72) at day 56, and pediatric AuCPCs, showing a similar rise from a 0.22-fold (± 0.14) to a 8.46-fold expression level (± 4.34) after 56 days of culture. Although non-significant, microtia AuCPCs increased their expression from 0.11-fold (± 0.10) at day 1 to 4.35-fold (± 1.55) at day 56.

In all groups, *COL10A1* (Figure 4D) was expressed at low levels compared to the housekeeping gene during culture. Its relative fold expression in adult AuCPCs was 0.01 (± 0.003) at day 1 and 0.28 (± 0.09) at day 56, whereas in pediatric AuCPCs there was a 0.04-fold (± 0.03) reduction at both timepoints. Microtia AuCPCs displayed a significant upregulation from a 0.05-fold (± 0.02) at day 1 to a 0.83-fold (± 0.43) reduction, relative to the housekeeping gene, at day 56. Similarly, *RUNX2* (Figure 4E) levels remained low in all groups. At day 1, the relative fold expression was 0.24 (± 0.05) in adult AuCPCs, 0.24 (± 0.16) in pediatric AuCPCs, and 0.31 (± 0.19) in microtia AuCPCs. Expression increased slightly yet non-significantly over time, with a 0.44-fold (± 0.14), a 0.57-fold (± 0.20) and a 0.48-fold (± 0.28) reduction in adult, pediatric and microtia AuCPCs respectively.

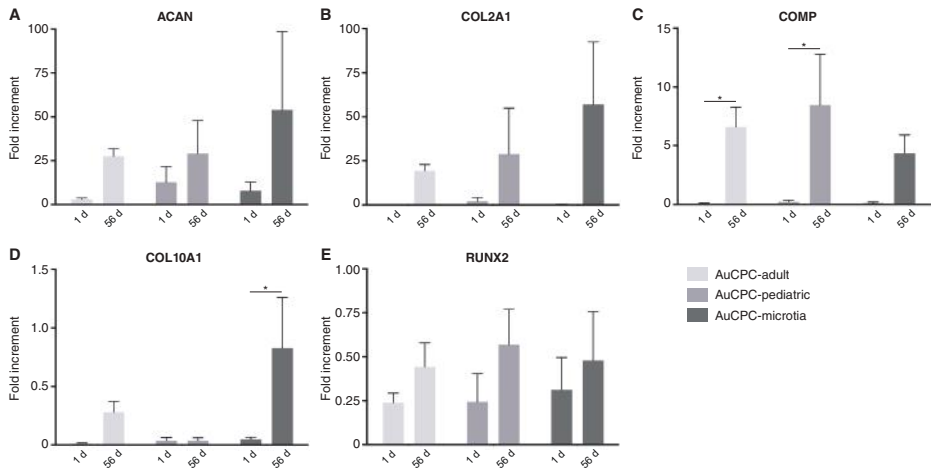

Figure 4. qPCR analysis of chondrogenic marker expression in cell-laden hydrogels. Relative gene expression of aggrecan (*ACAN*) (A), collagen type II (*COL2A1*) (B), cartilage oligomeric matrix protein (*COMP*) (C), collagen type X (*COL10A1*) (D) and runt-related transcription factor 2 (*RUNX2*) (E), normalized against housekeeping gene *HPRT1*. Statistically significant differences of $p < 0.05$ are indicated with an asterisk (*).

Chondrogenic culture of cell-laden hydrogels results in cartilage-specific matrix production

The synthesis of cartilage-specific matrix in cell-laden hydrogel constructs was assessed by the quantification of sulphated glycosaminoglycans (sGAG), which is representative of the proteoglycan content present in the neo-tissue. All groups demonstrated a significant increase in sGAG per dsDNA content during culture (Figure 5A), confirming chondrogenic differentiation and neocartilage production.

Adult AuCPCs showed a significant increase in sGAG from 1.07 µg/µg (± 0.23) at day 1, to 18.29 µg/µg (± 1.04) at day 28 and 31.52 µg/µg (± 1.44) at day 56. A significant sGAG production was also observed in microtia AuCPCs: 1.03 µg/µg (± 0.09) at day 1, 19.97 µg/µg (± 3.31) at day 28 and 33.23 µg/µg (± 1.84) at day 56 of culture. AuCPCs sourced from pediatric tissue exhibited the highest sGAG values with 0.51 µg/µg (± 0.12) at day 1 significantly increasing to 31.99 µg/µg (± 6.30) at day 28 and then further to 39.51 µg/µg (± 6.93) at day 56.

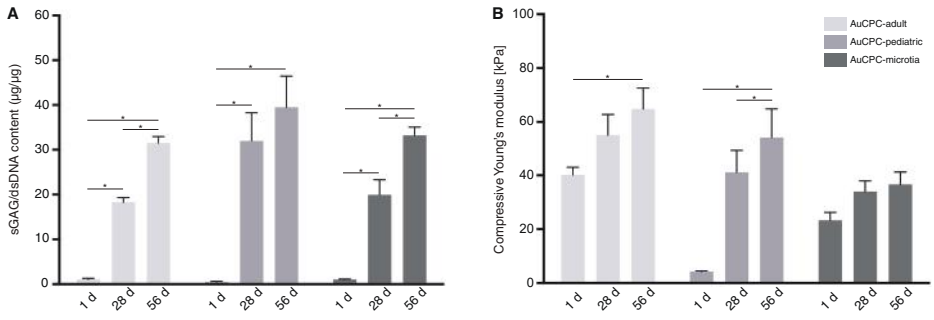

Figure 5. Biochemical composition and compression modulus of cell-laden hydrogels. Quantified sulphated glycosaminoglycan (sGAG) per dsDNA content after 28 and 56 days of chondrogenic culture, normalized against dry weight (A). Compressive Young's modulus as a measure of construct stiffness of cell-laden hydrogels after 28 and 56 days of culture (B). Statistically significant differences of $p < 0.05$ are indicated with an asterisk (*).

4

Hydrogel constructs display increased compressive properties over time

The compression modulus is representative of the stiffness of the cell-laden hydrogel constructs in terms of compression. The modulus increased in all groups during the culture period (Figure 5B). The compressive Young's modulus significantly increased over time in constructs loaded with adult and pediatric AuCPCs. Adult AuCPC samples exhibited a modulus of 55.09 ± 7.67 kPa at day 28 and 64.82 ± 7.73 kPa at day 56, of which the latter is a significant increase compared to day 1 (40.16 ± 2.87 kPa). Samples with pediatric AuCPCs started at a lower compressive Young's modulus at day 1 (4.25 ± 0.26 kPa) and increased significantly at both timepoints (41.15 ± 8.13 kPa at day 28 and 54.19 ± 10.66 kPa at day 56). There was a non-significant increase in compressive strength in microtia AuCPC samples over time, with a modulus of 23.29 ± 2.92 kPa at day 1, 34.00 ± 3.97 kPa at day 28 and 36.62 ± 4.61 kPa at day 56.

Cartilage-specific matrix deposition is confirmed by histology and immunohistochemistry

The presence and distribution of several components specific for auricular cartilage, including proteoglycans, collagens type II and I, as well as elastin, were visualized on histological sections. The stainings confirm neocartilage matrix deposition in hydrogels loaded with human AuCPCs.

Synthesized proteoglycans, as indicated by Safranin O staining, were most abundant in pediatric AuCPCs, followed by adult AuCPCs. There was an inhomogenous distribution of stained proteoglycans in adult AuCPC samples, with dense labelling in the pericellular territory gradually dispersing into the hydrogel (Figure 6A). Pediatric AuCPCs displayed an intense homogenous staining throughout the sample, with no observable increase between day 28 and day 56 (Figure

6B), corresponding to the quantified sGAG content. Microtia AuCPC samples exhibited isolated pericellular staining at day 28, with increasing distribution into the inter-territorial areas at day 56 of culture (Figure 6C).

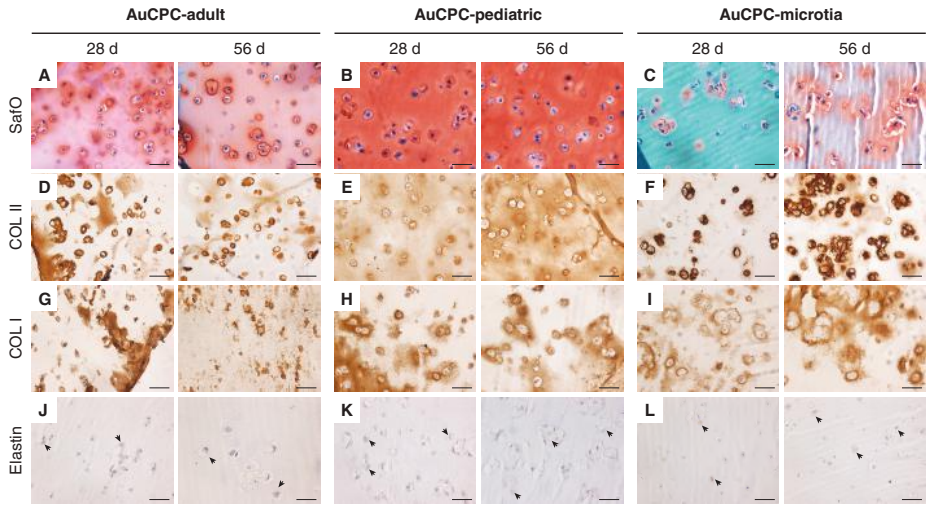

Figure 6. Histological analysis of cell-laden hydrogels after 28 and 56 days in chondrogenic culture. Safranin O staining visualizing proteoglycan deposition in adult (A), pediatric (B) and microtia (C) AuCPCs. Immunohistochemistry for collagen type II (D/E/F), collagen type I (G/H/I) and elastin (J/K/L). Black arrows indicate places of intracellular elastin staining. Scale bars equal 50 µm.

The deposition of collagen type II and collagen type I was predominantly localized in a broad peripheral area of the hydrogel sample. Collagen type II appeared concentrated pericellularly with clusters of intense brown staining in samples with adult and microtia AuCPCs (Figures 6D and 6F respectively). Pediatric AuCPCs displayed less intense staining, yet with a more widely distributed organization of collagens into the inter-territorial region, with a more intense staining observed pericellularly (Figure 6E). At day 56, microtia AuCPCs displayed the most intense collagen type II staining, corresponding to the mRNA expression profiles. Staining for collagen type I was generally less pronounced compared to collagen type II (Figure 6G-I). In all groups, staining remained localized in a wide territorial area and intensified slightly over time.

Elastin is a specific component of elastic auricular cartilage. All groups displayed an intracellular staining for elastin (Figure 6J-K), which increased over time in intensity and abundance. Staining was most apparent in samples containing pediatric AuCPCs, followed by microtia and adult AuCPCs.

DISCUSSION

The origin of the cells used for the generation of elastic cartilage-like tissue is an essential factor in determining the success of tissue-engineered auricular implants. However, the limitations of currently used cell sources hamper the development of high-quality engineered tissue constructs. This study identified cartilage progenitor cells in adult, pediatric and rudimentary microtia auricular cartilage and confirmed their potency for cartilage tissue engineering applications. This novel cell source has the potential to improve the quality and clinical feasibility of tissue-engineered auricular implants, facilitate the successful translation of the technology towards the clinic, and advance microtia reconstruction towards a less invasive technique.

Thus far, auricular cartilage tissue engineering strategies have mainly involved chondrocytes and mesenchymal stromal cells from various sources [26, 27, 48, 66, 116, 136, 153, 155, 169, 171, 185, 200, 240, 291, 292, 294, 306, 349, 374, 382, 387]. An advantage of using primary chondrocytes is that the cells are mature and thus exclusively committed to the chondrogenic lineage. However, chondrocytes maintain a low proliferative capacity and are known to dedifferentiate in monolayer culture due to continuous multiplication, passaging and low seeding densities, shifting towards a fibroblast-like phenotype and corresponding matrix production that is lacking the biochemical and biomechanical properties of native elastic cartilage [25, 63, 144, 189, 210, 234, 307, 310]. Higher cell seeding densities in proliferative culture may reduce dedifferentiation, but obtaining sufficient chondrocytes for clinically relevant tissue-engineering purposes still remains a significant challenge [210]. The engineering of a human-sized auricle would require between 100 and 250 million cells, depending on implant volume and seeding density [21, 25, 66]. Approximately 2 million chondrocytes can be harvested from a non-deforming biopsy from the human auricle [210], which can only be expanded to roughly 10 million cells before undesirable phenotypic changes occur [21]. Mesenchymal stromal cells, on the other hand, are readily available in bone marrow and adipose tissues. Due to their self-renewal capacity, they can be expanded extensively whilst remaining a stable phenotype [290]. Their multi-lineage differentiation ability includes chondrogenesis, which has been applied successfully in cartilage tissue engineering studies [2, 59, 95, 117, 280, 292, 313, 359, 387]. Nevertheless, their tendency to undergo hypertrophic differentiation can result in calcified cartilage serving as a remodeling template for bone mineralization through the endochondral ossification pathway [120, 229]. Cartilage calcifications after auricular reconstruction are undesirable as they can lead to loss of flexibility, increased stiffness, an unnatural feel of the reconstructed ear, patient discomfort, and potential risk of implant fracture or extrusion through the skin [25, 173, 257].

Cartilage progenitor cells (CPC), a new player in cartilage tissue engineering, can be sourced from an autologous auricular cartilage biopsy and can be readily expanded to high cell numbers while

maintaining chondrogenic differentiation capacity in an inducive environment. CPCs are a resident subpopulation of cartilage cells that – in contrast to primary chondrocytes – exhibit stem cell-like properties. These cells were first identified in the superficial zone of articular cartilage [86], and subsequentially also in the cartilage of the auricle [265, 377]. Distinctive properties of CPCs include their ability to form large colonies from an initially low seeding density [86], the expression of the putative stem cell markers *CD73*, *CD90* and *CD105* [84, 368] as well as the fibronectin receptor *CD49e* [368], and the retainment of multi-lineage differentiation potential [368]. These factors discern this specific subpopulation of cartilage-resident stem/progenitor cells from other cell samples that are frequently named chondroprogenitors – a term often used for any progenitor cell driven towards the chondrogenic lineage [157]. The CPC subpopulation comprises 0.1-1 % of the total cell content of cartilage and can be isolated through fibronectin adhesion [86, 368]. The presence of fibronectin-adhering cartilage progenitor cells in the auricular cartilage has previously been confirmed in porcine and equine species [265, 377]. This is the first study to isolate these cartilage progenitor cells from human auricular cartilage and apply these cells for tissue engineering purposes.

The presence and functionality of CPCs from adult, pediatric and even microtia cartilage sources provides feasible options for clinical application of cell-based auricular reconstruction strategies. Instead of requiring a large donor site in the case of chondrocytes, or a bone marrow aspirate for MSCs, auricular CPCs can be easily obtained from the rudimentary cartilage in microtia or through a non-deforming biopsy from the normal external ear. This would yield between 2 and 20 thousand AuCPCs, which can then be expanded and passaged multiple times, generating over 250 million cells in only 11 to 17 population doublings. Following our results on the proliferation capacity of human AuCPCs, with rates ranging from 0.43 to 1.49 population doublings per 24 hours, these cell numbers could be attainable within one to six weeks of *in vitro* culture and in less than 5 passages. During this time, AuCPCs do not lose their capacity for multilineage differentiation. This study demonstrated the ability of AuCPCs to differentiate towards bone, adipose tissue and cartilage after 3, 4 and 5 passages. Chondrogenic matrix deposition remained abundant among donor sources and over time; only adult AuCPCs, which were sourced from elderly donors, displayed diminished cartilage production at passage 5.

In accordance with the standard definition for MSCs [84], AuCPCs are plastic adherent and demonstrate the potential to differentiate into multiple lineages. Further, ≥95 % of the putative stem cell population must express surface antigens specific to *CD90*, *CD105* and *CD73*, which partially applies to the AuCPCs found in our study. Given our analysis, human AuCPCs qualify in terms of *CD105* and *CD73* expression but defer from the standard definition of MSCs in the case of *CD90* expression. However, this set of markers has been specifically developed to distinguish MSCs from other stem cell types (*i.e.* hematopoietic stem cells in the bone marrow) and may not fully match

the profile of other mesenchymal progenitor cells present in different tissues. To date, there is no unique set of markers identified for the selection of CPCs derived from articular cartilage (the most studied source of chondroprogenitors), and even less is known about auricular cartilage-resident progenitor cells [265, 377, 385]. Yet, some preliminary studies are starting to indicate potential markers to distinguish articular chondroprogenitors, such as co-expression of *CD166* and *STRO-1* [4, 360], which may be useful for auricular progenitors as well. Regardless, plastic adherence, colony formation, abundant proliferative abilities and multipotent differentiation capacity are stem cell-associated properties that are highly beneficial for tissue engineering purposes.

Besides a potent cell source, successful cartilage tissue engineering requires an appropriate microenvironment for maturing cells to thrive in. Specifically, a three-dimensional (3D) environment is a key element in supporting the chondrogenic potential of cells, thereby fostering a cartilage-like gene expression profile and corresponding extracellular matrix production [270]. Hydrogels are especially suitable as cell carriers, being highly hydrated porous polymer networks that can provide a permissive 3D environment for chondrogenic differentiation and neocartilage formation [357]. Gelatin methacryloyl (gelMA) has proven to be a favorable choice for cartilage tissue engineering strategies due to its biocompatibility, natural bioactivity, and tailorability [177]. This hydrogel system has previously been shown to be conducive for chondrogenesis [193, 194, 312] and to support equine auricular and articular CPCs in producing cartilage-like matrix *in vitro* [193, 265]. Similarly, the human AuCPCs in this study demonstrated evident chondrogenic potential in gelMA constructs. At the genetic level, the differential expression of markers for aggrecan, collagen type II and cartilage oligomeric matrix protein all increased over the 56-day culture period. Biochemical analysis confirmed the synthesis of cartilage-like matrix in hydrogels seeded with AuCPCs.

Proteoglycans are the major structural components of cartilage. The quantification of glycosaminoglycans showed a significant increase of GAG per DNA over time in all groups, indicating abundant neotissue matrix synthesis. The conglomeration of proteoglycans contributes to the mechanical properties of cartilage tissue. Corresponding with the biochemical results, a significant increase in compressive modulus over time was found in samples laden with adult and pediatric AuCPCs; however, this was not the case for microtia AuCPCs. Histological evaluation may provide an explanation for this observation. Cartilage-specific matrix deposition in cell-laden hydrogels was evident in all constructs laden with adult, pediatric or microtia AuCPCs. Nevertheless, pediatric AuCPCs exhibited a homogenous distribution of synthesized matrix components throughout the hydrogel, whereas the deposition of proteoglycans and collagens by microtia AuCPCs remained predominantly in the pericellular to territorial matrices. As the specific organization of a tissue impacts its mechanical properties [372], the nonsignificant changes in compressive modulus in constructs with microtia cells may be attributed to this inhomogeneous

cluster-like organization. In contrast, the more homogenous incorporation of proteoglycans in the extracellular matrix by adult and pediatric AuCPCs appears to contribute significantly to the increasing compressive properties of the constructs. The compression moduli achieved in this study by encapsulating human AuCPCs in gelMA, ranging from 36.6 to 64.8 kPa after 56 days of culture, are markedly lower than the native situation. Various biomechanical properties of native auricular cartilage have been reported to be at least in the MPa range [130, 255], demanding tissue-engineered constructs to be structurally enhanced with supporting frames [54, 361] or with a more refined reinforcing fiber network [170, 217, 363]. Such strategies can mechanically support engineered constructs during *in vitro* and *in vivo* maturation of the neotissue.

Specific to auricular cartilage is the presence of a network of elastin fibers. Elastin is critical for the long-term function of the auricular cartilage and the maintenance of its shape, as this biopolymer is stable, durable and allows elastic recoil and resilience of the tissue [223]. The development of elastic fibers is slower compared to other cartilage matrix components [66]. In studies applying auricular chondrocytes or a chondrocyte-MSC co-culture in a pellet or hydrogel system, elastin fibers started appearing after 6-12 weeks of *in vivo* culture [21, 26, 45, 66, 142, 356]. Although the specific requirements for elastin formation are still largely uncertain, it has been suggested that *in vitro* culture alone is insufficient [142]. In a study by Hellingman *et al.* (2011), an absence of elastin was observed after 10 weeks *in vitro* culture of pelleted auricular chondrocytes, whereas implanted samples demonstrated elastin production after 6 weeks *in vivo* [142]. Yet, our study demonstrated intracellular expression of elastin by AuCPCs after 8 weeks of *in vitro* culture in gelMA. The supplementation of the differentiation media with TGF-β1, known for its ability to stimulate the expression of tropoelastin [223], may be a contributing factor to this observation. Another explanation may be the inherent potency of AuCPCs to reproduce their native environment, *i.e.* the elastic cartilage of the auricle.

The bending properties of the ear are integral for withstanding the daily external influences on the auricular structure. Hence, stimulating the formation of elastic fibers in tissue engineered cartilage for the auricle is necessary, yet the importance of preventing the formation of calcifications should not be overlooked. Mineralization of the neotissue may lead to construct stiffness, shape distortion and potentially implant fracture or extrusion [159]. As such, avoidance of cellular hypertrophy and subsequent tissue mineralization is essential. A marker of chondrocyte hypertrophy is collagen type X [213], whereas *RUNX2* is a marker of osteogenic differentiation [182]. Collagen type X is actually present in native non-mineralized auricular cartilage [76, 123, 142, 272] and its expression without subsequent mineralization has been reported in several studies applying chondrocytes for cartilage tissue engineering [76, 142]. In our study, the mRNA expression of *COL10A1* was low in adult and pediatric AuCPCs, however its expression was upregulated in microtia AuCPCs. In addition, a non-significant upregulation of *RUNX2* was observed in all groups during the 8-week

culture period. Compared to markers more typical of mature cartilage, *i.e.* aggrecan, collagen type II and cartilage oligomeric matrix protein, the expression levels of *COL10A1* and *RUNX2* are very low. Our previous study using equine AuCPCs showed similar expression levels of these markers without mineralization of the neotissue, as confirmed by histology [265]. Nevertheless, maintenance of the chondrogenic phenotype should be monitored for human AuCPCs in future studies, during *in vitro* culture and even more so during *in vivo* application.

As microtia is a developmental disorder associated with genetic aberrations [203], cells sourced from rudimentary microtia cartilage may have different properties than those from normal cartilage. Microtia cartilage has a more disorganized microscopic appearance, yet gene expression profiles and biochemical composition are similar to normal auricular cartilage [133, 152, 220]. There are only a few studies that have compared microtia chondrocytes to healthy human chondrocytes when applied for tissue engineering purposes, and although the majority found them to synthesize similar neocartilage tissue *in vivo* [152, 153, 169, 240], contrasting results have been reported. A recent comparison describes higher GAG content, higher Young's modulus, and higher cartilage-specific gene expression by healthy chondrocytes [133]. Our study is the first to report on cartilage progenitor cells sourced from the rudimentary microtia cartilage and our results indicate the ability of these cells to synthesize new cartilage tissue in an *in vitro* 3D hydrogel system. Compared to healthy adult and pediatric AuCPCs, cells from microtia cartilage seem to perform somewhat differently in terms of matrix organization and gene expression levels. Their aberrant origin remains a point of further investigation, focusing on genetic profiles and regenerative behavior in the long-term. Nevertheless, the rudimentary microtia cartilage can be a very valuable source of potent cartilage-producing cells, obviating the need for biopsies in healthy tissues.

Another important observation in this study is the variability between individual donors. This is a well-known challenge in cells and tissues originating from human sources [327]. When evaluating group averages, donor variance can be reflected in the standard deviation, yet this may impair the statistical analysis when comparing different groups. For improved insight in the regenerative response, it may be useful to correlate the results of each donor individually. Although in our study most donors exhibited substantial regenerative potential, we found some donors to underperform, thereby affecting average group results and statistical outcomes. Donor-to-donor variance may be linked to age, gender and disease [316, 329]. Nevertheless, our results show that even in case of microtia, potent and regeneration-competent cells are residing in the tissue. It would be advisable to start assessing individual donor performance and to subsequently determine the factors that can predict satisfactory outcomes. In the end, personalized medicine ultimately requires the definition of a set of quality control markers to benchmark whether the harvested cells are good enough to use for tissue-engineering applications for that patient.

Surgical correction of auricular deformities can greatly enhance a patient's psychosocial functioning and quality of life. The current state-of-the-art treatments bring meaningful change, yet have donor site morbidities, absence of a natural feel, and in case of foreign material the chance of implant extrusion. Therefore, improved reconstruction strategies are desired. The tissue engineering approach using autologous cells and bioresorbable supporting materials could provide a long-term solution, by essentially regenerating native-like tissue with appropriate properties. Challenges remain in obtaining sufficient autologous cells and subsequently generating high quality neotissue, yet the availability of a potent progenitor subpopulation in the human auricular cartilage presents encouraging opportunities for the successful engineering of the human auricle and its translation towards the clinic.

CONCLUSION

Human auricular cartilage, including the rudimentary microtia remnant, harbors potent fibronectin-adhering progenitor cells with high proliferative qualities and evident chondrogenic differentiation capacity. These cartilage progenitor cells thus have the ability to supply the required cell numbers for tissue engineering of an auricular implant, while maintaining the chondrogenic phenotype and producing cartilage-like neotissue in a 3D hydrogel system. These cells from this newly identified source can be easily obtained through a non-deforming biopsy of the normal ear or from the rudimentary microtia cartilage and can provide an important solution to long-existing challenges in auricular cartilage tissue engineering.

AUTHOR AFFILIATIONS

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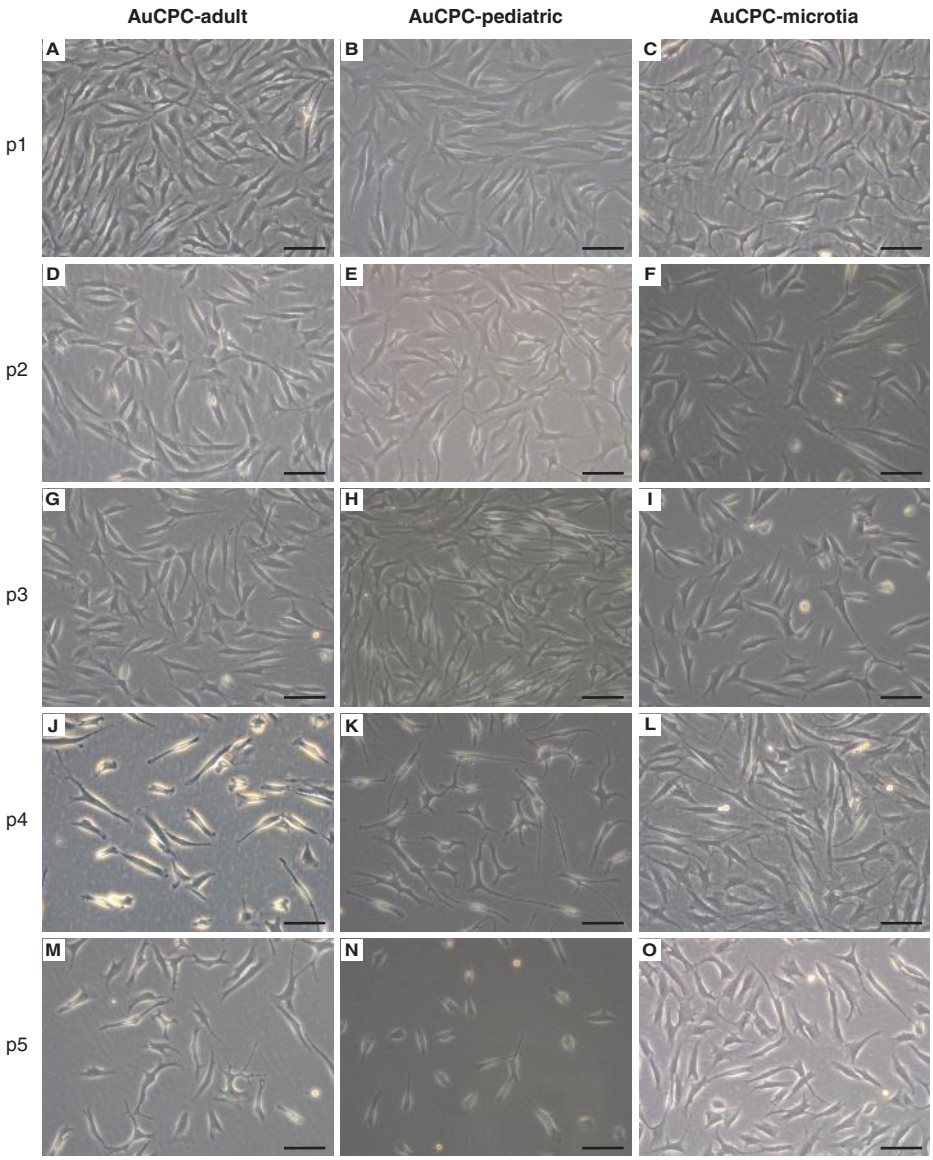
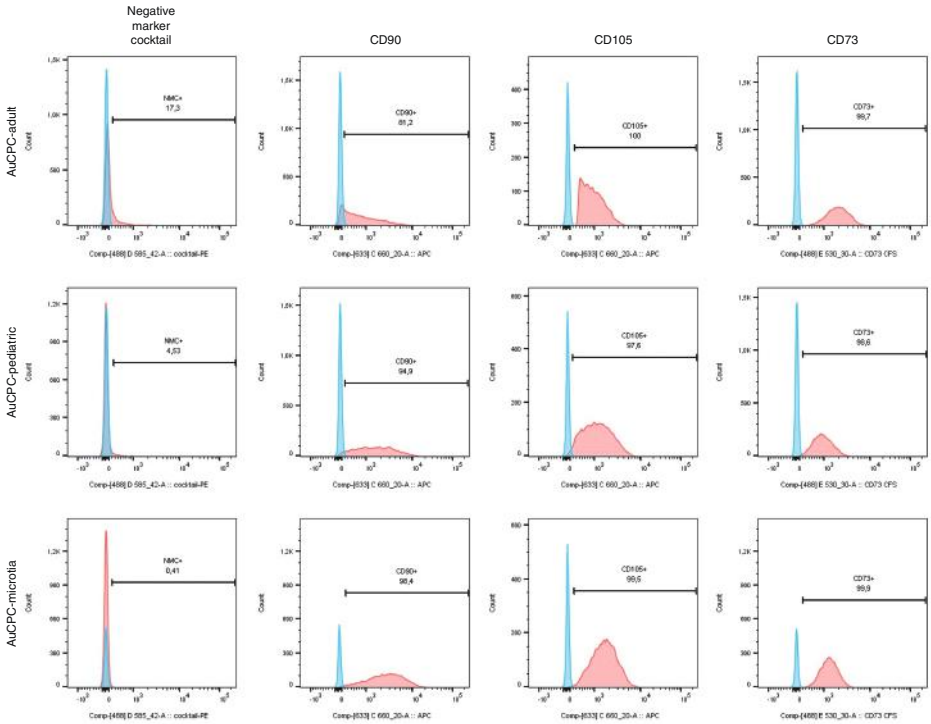
SUPPLEMENTARY INFORMATION

Supplementary Table S1. Primer sequences applied for qPCR evaluation of cartilage-like tissue synthesis. Forward (Fw) and reverse (Rv) primers of human aggrecan (*ACAN*), collagen type II (*COL2A1*), collagen type X (*COL10A1*), cartilage oligomeric matrix protein (*COMP*) and runt-related transcription factor 2 (*RUNX2*), as well as the housekeeping gene hypoxanthine phosphoribotransferase 1 (*HPRT1*). Expected amplicon sizes are expressed in base pairs (bp).

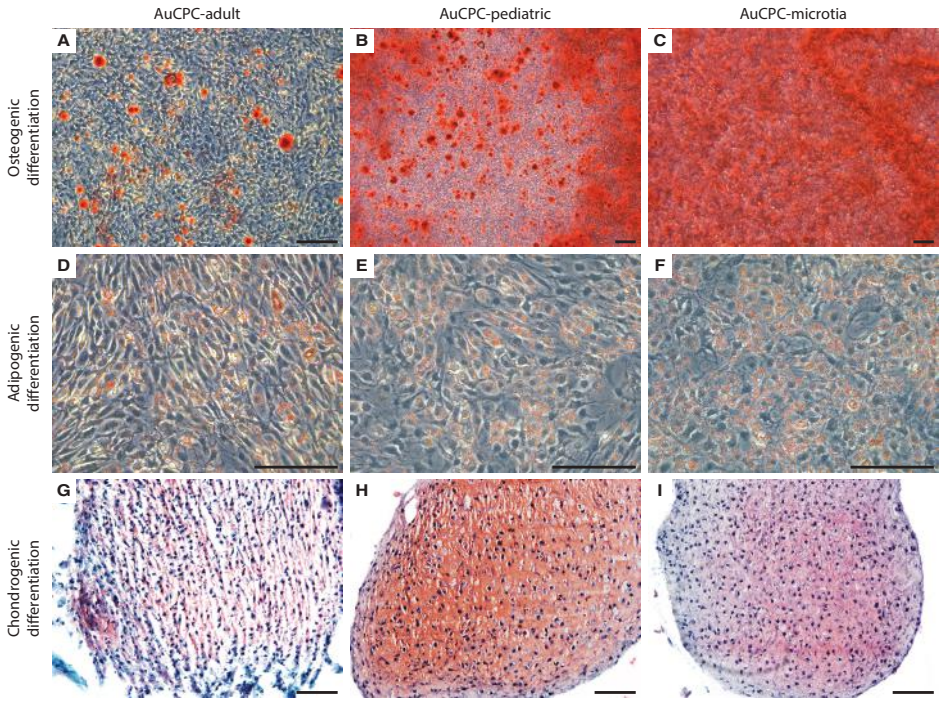
Target gene		Primer sequence (5' to 3')	Amplicon size (bp)
HPRT1	Hypoxanthine phosphoribotransferase 1	Fw: TATGGACAGGACTGAACGTCTTG	76
		Rv: CACACAGAGGGCTACAATGTG	
ACAN	Aggrecan	Fw: ATGTTCCCTGCAATTACCACCT	121
		Rv: TTGATCTCATACCGGTCCTTCTTC	
COL2A1	Collagen type II, α1 chain	Fw: GCCTCAAGGATTTCAAGGCAAT	132
		Rv: GCTTTTCCAGGTTTTCCAGCTT	
COL10A1	Collagen type X, α1 chain	Fw: CAAGGCACCATCTCCAGGAA	70
		Rv: AAAGGGTATTTGTGGCAGCATATT	
COMP	Cartilage oligomeric protein	Fw: CCCCAATGAAAAGGACAACTGC	121
		Rv: GTCCTTTTGGTCGTCGTTCTTC	
RUNX2	Runt-related transcription factor 2	Fw: TTACAGTAGATGGACCTCGGGA	104
		Rv: AGGAATGCGCCCTAAATCACT	



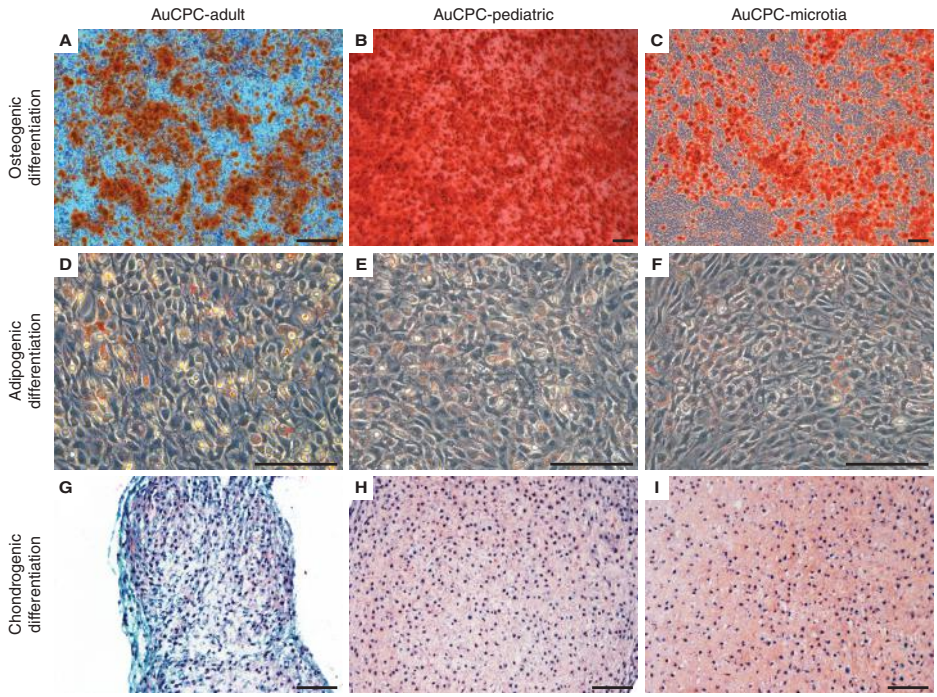
Supplementary Figure S1. Morphological features of AuCPCs captured at various passages. Adult, pediatric and microtia AuCPCs all displayed a polygonal and spindle-shaped morphology that was maintained from passage 1 up to passage 5 (A-O). Scale bars equal 50 µm.



Supplementary Figure S2. Histograms corresponding to flow cytometry analysis. High expression levels of *CD90, CD105* and *CD73* were observed in adult, pediatric and microtia AuCPCs. Low expression of the negative marker panel was detected in most populations. Each row of histograms displays a representative donor. An appropriate isotype control for each antibody was used.



Supplementary Figure S3. Trilineage differentiation capacity of AuCPCs in passage 3. Upon stimulation with appropriate differentiation media, AuCPCs in passage 3 demonstrated the ability to produce mineralizations (A/B/C), lipid vesicles (D/E/F) and proteoglycans (G/H/I). Scale bars equal 100 µm.



Supplementary Figure S4. Trilineage differentiation capacity of AuCPCs in passage 5. Upon stimulation with appropriate differentiation media, AuCPCs in passage 5 demonstrated the ability to produce mineralizations (A/B/C), lipid vesicles (D/E/F) and proteoglycans (G/H/I). Scale bars equal 100 µm.

PART II

FABRICATION

IN SEARCH OF A DURABLE AURICULAR IMPLANT SHAPE

CHAPTER 5

ACCURATE MEASUREMENTS OF THE SKIN SURFACE AREA OF THE HEALTHY AURICLE AND SKIN DEFICIENCY IN MICROTIA PATIENTS

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ABSTRACT

The limited cranial skin covering auricular implants is an important yet underrated factor in auricular reconstruction, for both reconstruction surgery and tissue engineering strategies. We report exact measurements on skin deficiency in microtia patients and propose an accessible pre-operative method for these measurements. Plaster ear models (n = 11; M:F = 2:1) of lobular-type microtia patients admitted to the University Medical Center Utrecht in The Netherlands were scanned using a micro-CT or a cone-beam CT. The resulting images were converted into mesh models from which the surface area could be calculated. The mean total skin area of an adult-size healthy ear was 47.3 cm^2, with 49.0 cm^2 in men and 44.3 cm^2 in women. Microtia ears average 14.5 cm^2, with 15.6 cm^2 in men and 12.6 cm^2 in women. The amount of skin deficiency was 25.4 cm^2, with 26.7 cm^2 in men and 23.1 cm^2 in women. This study proposes a novel method to provide quantitative data on the skin surface area of the healthy adult auricle and the amount of skin deficiency in microtia patients. We demonstrate that the microtia ear has <50 % of skin available compared to healthy ears. Limited skin availability in microtia patients can lead to healing problems after auricular reconstruction and poses a significant challenge in the development of tissue engineered cartilage implants. The results of this study could be used to evaluate outcomes and investigate new techniques with regard to tissue-engineered auricular constructs.

BACKGROUND

Microtia is a congenital malformation of the external ear, characterized by underdevelopment of the auricle, ranging from a slight reduction in size to a peanut-like lobular structure or its complete absence [141, 204, 354]. The prevalence of microtia depends on ethnicity and region, with an overall prevalence of 1.55 per 10,000 births (CI 1.50-1.60), and with lobular-type microtia being the most frequent type [204]. Associated craniofacial abnormalities include auditory canal atresia, middle ear dysplasia, mandibular hypoplasia, facial cleft or facial asymmetry [3, 141].

There are various options for the treatment of microtia, including osseointegrated prostheses and alloplastic implants such as Medpor® [243, 274, 298, 319]. Currently, surgical reconstruction of the auricle using autologous costal cartilage is most often performed [25, 40, 65, 151, 199]. The carved framework is in fact considerably thicker and less pliable than natural cartilage in order to maintain shape and detail of the implant when covered with the thick cranial skin. The skin poses some important but often overlooked challenges in auricular reconstruction, among which limited skin availability and contractive forces on the implant [17, 199, 389].

The skin is a highly viscoelastic tissue and therefore has high mechanical restraining capabilities [91, 149]. Elastin and collagen are among the structural components ensuring tensile strength and extensibility. With increasing strain, the skin offers more resistance and presses the underlying material. These contraction forces may lead to healing problems following auricular reconstruction [17, 389]. This is especially evident in microtia patients, who may have only limited skin available [319].

The same problem arises in regenerative approaches for engineering an auricular implant. Although many advances have been achieved in ear-shaped cartilage regeneration, a major challenge is the maintenance of size and shape of the relatively large complex-shaped three-dimensional (3D) construct after implantation. The covering skin applies a great deal of pressure on the neocartilage implant, which initially lacks adequate mechanical stability to withstand such forces. With less skin available, these forces will increasingly hamper the development of the auricular construct [25].

To generate sufficient skin coverage of the implanted framework, in auricular reconstruction, tissue expansion, flap transposition and skin grafts can be utilized [178, 199, 381]. There is very limited information in the literature on the actual amount of skin in the normal ear. Yazar *et al.* calculated the area of skin covering the healthy human auricle in a Turkish population [378]. More relevantly, no data is available on skin surface area of microtia ears, leaving the shortage of skin that must be compensated for with *e.g.* skin grafting an educated guess.

This retrospective study addresses these issues and provides quantitative data on skin surface area of both healthy and microtia ears in humans, with specific interest in the amount of missing skin for adequate coverage of an auricular implant. In addition, we present an accessible method to assess skin requirements pre-operatively in patients with auricular deformations. Moreover, this method may be especially interesting as an evaluation tool for size evaluation after reconstruction or analysis of a tissue-engineered auricular implant.

MATERIALS & METHODS

Patient demographics

Plaster ear models of microtia patients admitted to the University Medical Center Utrecht (UMCU), the Netherlands, have been collected between 1999 and 2005. Microtia ears of the lobular type were selected for this study and compared to their contralateral normal counterparts. The lower age limit of 9 years was chosen based on the age at which the majority of auricular reconstructions are performed [40], coinciding with the average age of maturity of the ear [94, 317]. Due to anonymous plaster model analysis, the institutional review committee required no ethical approval.

Computed tomography scanning of plaster ear models

Plaster models were scanned using a micro-computed tomography scanner (μCT; Quantum FX, Perkin Elmer, USA; tube voltage 90 kV; tube current 180 μA; scan time of 17 sec; voxel size of 0.146 x 0.146 x 0.146 mm^3) or a cone-beam computed tomography scanner (CBCT; Next Generation, i-Cat; voxel size of 0.250 x 0.250 x 0.250 mm^3) depending on the size of the models. The CBCT scanner yielded DICOM (Digital Imaging and Communication in Medicine) images. The images from the μCT were converted into DICOM files as well using Analyze 11.0 (MayoClinic, USA).

Creation of digital 3D models

Volumetric data of the plaster models were extracted from the scans with Matlab R2013a (The Mathworks Inc., USA) using a threshold technique, which defines the volumetric data as every pixel above a certain threshold value. The isosurface, *i.e.* the three-dimensional surface that represents the points of the constant value, was subsequently computed from the volumetric data and exported into STL-files, known as mesh models. A mesh model is a representation of the surface of the original plaster model. They are made up of small connecting triangles (faces), defined by coordinates in a three-dimensional grid (vertices). Each face has its own surface area and the accumulated areas of all faces will provide an accurate measurement of the surface are of the plaster model (Figure 1).

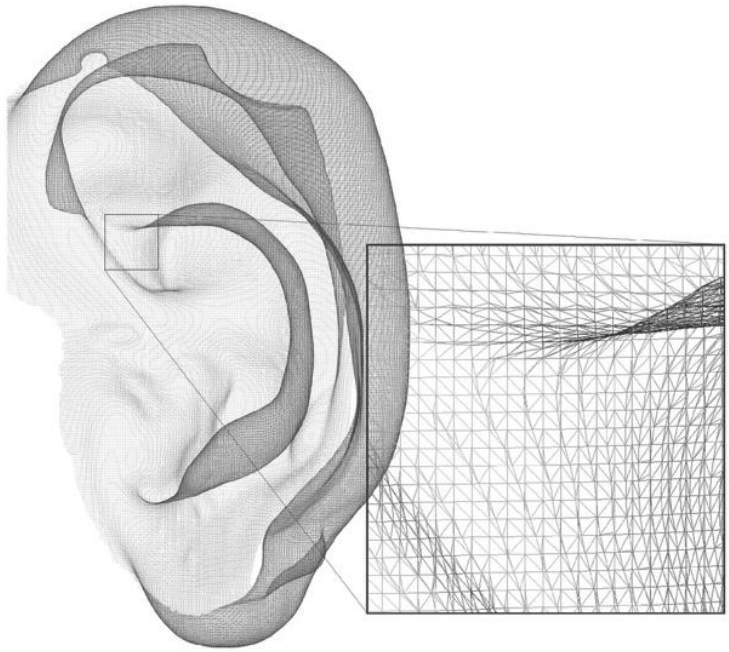

Figure 1. Mesh model based on CT scan of the auricular plaster model. The surface of the original model is represented by small connecting triangles (faces), which all have their own surface area. Accumulation of these areas provides an accurate measurement of the total surface area of the model.

Surface area calculation

The surface area calculation was subsequently performed using MeshLab (Visual Computing Lab, Italy), which is a 3D mesh processing system. By computing the geometric measures, a surface in square millimeters (mm^2) was obtained. The calculation for the auricular surface area itself differs from the calculation of the skin deficiency, as these require different area boundaries of the models.

Determination of the auricular surface area

The first objective of this study is to determine the exact auricular surface area. Therefore, the boundaries of the area included in the calculations were set at the curvature where the auricle joins the cranium. The external ear, including the lobe, was subsequently cut out at its base by means of a drawing tool (Figure 2). This approach will be referred to as the 'base method' throughout this chapter.

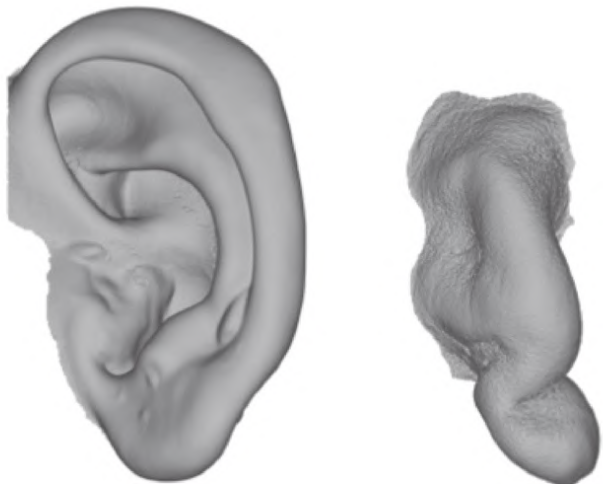

Figure 2. 'Base method' for calculation of auricular surface area. Boundaries used in the base method, where the ear model is cut out at its base for calculation of the auricular surface area of the healthy adult ear.

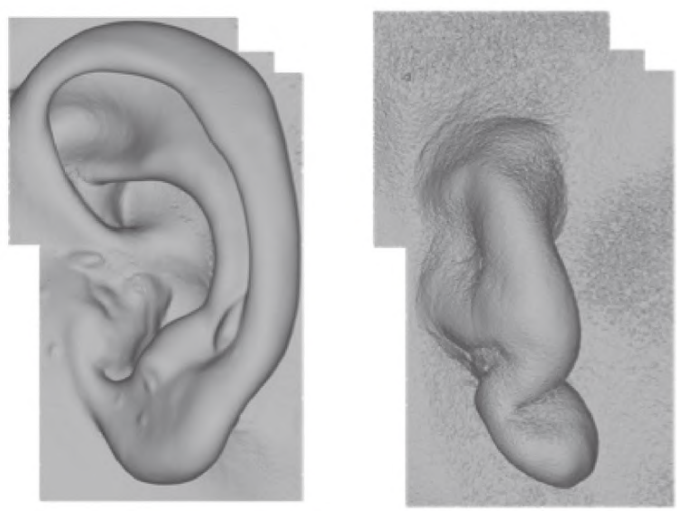

Figure 3. 'Fixed method' for calculation of differences between ears. Boundaries used in the fixed method, where a fixed domain around the respective ears enables quantification of skin deficiency.

Determination of the amount of skin deficiency in microtia ears

The second objective is to determine the amount of skin deficiency for auricular reconstruction in microtia patients. The difference between the surface area of the healthy ear and the microtia ear equals the amount of skin missing to cover an implant with the same surface as the healthy contralateral ear. However, because of the differences in shape, there is a discrepancy in base areas of the healthy ear and the microtia ear. Therefore, a fixed domain around both ears was selected (Figure 3) in order to eliminate such confounding factors. Comparison of identical domains will allow objective calculation of the difference in skin surface areas, and thus the determination of the amount of skin deficiency. This approach will be referred to as the 'fixed method'.

Statistical analysis

Calculations of the mean and standard deviation (SD) of the surface area were performed using Microsoft Excel (Microsoft Corporation, USA). In order to evaluate the validity of our method, the results were compared to the only comparable study [378]. Difference significance in comparison to this previous study was calculated using a Welch t-test in GraphPad (GraphPad Software, USA). Difference significance between women and men was calculated using the Mann Whitney u-test in SPSS (IBM, USA). A significant difference was defined as $p < 0.05$.

RESULTS

Patient demographics

Eleven patients with lobular-type microtia were included in this retrospective study (M:F = 2:1). Patients were between 9 and 52 years old at the time of the first reconstruction surgery, with a mean of 26 years and a median of 22 years (male: mean 27 years, median 22 years; female: mean 24 years, median 20 years).

Surface area of the auricle

The exact auricular surface area of a healthy adult-size human ear, as determined using the base method, was calculated to be 47.3 cm^2 (SD 4.4) overall, where men generally had larger ears (49.0 cm^2, SD 4.7) than women (44.2 cm^2, SD 1.6; $p = 0.073$; Table 1). Using this same method, microtia ears average 14.5 cm^2 (SD 4.0), with 15.6 cm^2 (SD 4.7) in men and 12.6 cm^2 (SD 1.9) in women ($p = 0.412$; Table 1).

Table 1. Mean skin surface area of the healthy and the microtia ear. Using the base method, where the auricle was cut out at its base, the exact auricular surface area was determined.

	Healthy ear (cm^2)	**Microtia ear** (cm^2)
Male (*n* = 7)	49.0 (SD: 4.7)	15.6 (SD: 4.7)
Female (*n* = 4)	44.2 (SD: 1.6)	12.6 (SD: 1.9)
Overall (*n* = 11)	47.3 (SD: 4.4)	14.5 (SD: 4.1)

Amount of skin deficiency in microtia ears

The difference between the surface area of the healthy ear and the microtia ear, as calculated using the fixed method, can be interpreted as the amount of skin missing to cover the auricular implant. The mean skin deficiency was 25.4 cm^2 (SD 4.6), with 26.7 cm^2 in men (SD 4.6) and 23.1 cm^2 in women (SD 4.1; $p = 0.315$; Table 2).

Table 2. Mean difference in skin surface area between healthy and microtia ear, as calculated using the fixed method. By selecting a fixed domain around both ears, an objective calculation of the difference in surface area was obtained. This difference can be interpreted as the amount of skin deficiency on the microtia side compared to the healthy ear.

	Difference (cm^2)	**Deficiency** (%)
Male (*n* = 7)	26.7 (SD: 4.6)	54.5
Female (*n* = 4)	23.1 (SD: 4.1)	52.3
Overall (*n* = 11)	25.4 (SD: 4.6)	53.1

DISCUSSION

Limited skin availability in microtia patients proves to be a problem in both surgical and regenerative medicine approaches for the reconstruction of the auricle. Skin expansion and skin grafting solutions are currently used to generate sufficient skin coverage of the reconstructed implant, yet the actual amount of skin required for an implant to be adequately covered remains an educated guess. Meanwhile, novel tissue-engineering approaches to reconstruct the auricle are hampered in several ways [25]. One of the main problems is that the construct does not keep its shape under the tight skin envelope [389]. Although we fully agree that mechanical properties of the tissue-engineered auricle should also be investigated [257], it seems imperative to objectively assess the amount of skin deficiency in the microtia patient.

This retrospective study used 3D scan images to calculate auricular surface area. The results indicate that the healthy human adult-size auricle averages 47.3 cm^2 overall, with 49.0 cm^2 in men and 44.3 cm^2 in women in our patient population. These numbers are comparable to a similar

study conducted by Yazar *et al.* (2013) [378]. Their study involved a technique based on measuring cut-out silicone impression models, conducted on a population of adult Turkish men and women. The skin area calculated using this technique was also determined with boundaries set at the curvature from auricle to skull, and by adding the mean anterior and posterior surface areas the data could be compared to the current study. The male population in the study by Yazar *et al.* (2013) exhibited a total skin surface area of 51,4 cm^2 (p = 0.23), whereas woman had quite smaller ears with 41,0 cm^2 (p = 0.03), averaging 46,3 cm^2 overall (p = 0.51) [378]. Overall, the auricular surface areas in both studies do not differ significantly, as expected. The significant difference in the female Turkish population may be explained by the small subject group in our study, or possibly an ethnical effect [309].

Calculating the exact auricular skin surface area and subsequently the amount of skin deficiency contributes to the general knowledge on the properties of the healthy adult auricle and may aid surgeons pre-operatively. The method we propose here to calculate the auricular surface area yields similar results as a previous study [378]. However, this base method is not appropriate for determining the amount of skin deficiency on the microtia side, as it does not take into account differences in the area where the auricle joins the skull, and there is a discrepancy in this base-area between microtia and healthy ears. In addition, the determination of the base borders is rather subjective, and even more challenging to define in an underdeveloped auricular structure. A more objective way to calculate the difference in skin area between the healthy and the microtia ear, as proposed in the current study, is by using a fixed border around both ears, which enables comparison between two identical domains. The subsequently calculated difference in surface area can be interpreted as the amount of skin missing for adequate coverage of a reconstructed auricular implant. Following this fixed method, our study indicates an average shortage of 25.4 cm^2 overall, with 26.7 cm^2 in men and 23.1 cm^2 in women. These numbers indicate a skin deficiency on the microtia side of more than 50 %.

The mechanical properties of the skin enable it to offer more resistance with increasing strain [149]. Stretching the skin over an auricular implant places increasing forces on the underlying material. The findings of this study indicate that there is a significant deficiency of skin on the microtia side, making the influence of the mechanical properties of the skin on the auricular implant a factor that should not be ignored in clinical practice. In regenerative medicine approaches, the contractive forces of the skin play an especially important yet often overlooked role as well. Previous experiments are mostly performed in murine models with relatively loose skin [389], contrary to the thick and stiff human cranial skin [149]. In microtia patients, where there is a loss of skin over the ear and mastoid area, the contractive forces will be even stronger. Tissue-engineered constructs may not be able to maintain their shape in the tight skin envelope in these patients. Providing extra skin through *e.g.* tissue expansion may be imperative to a successful tissue-

engineered implant. This study could provide an impetus for further research on regenerative medicine approaches to microtia and auricular reconstruction.

We have presented a reliable and simple method for the calculation of skin deficiency in microtia patients, one that is less time-consuming and labor-intensive than the method proposed in a previous study [378]. We believe that our method can easily be applied in clinical practice in preparation of auricular reconstruction or for evaluating post-reconstruction aesthetic outcomes, yet it may be even more interesting as an evaluation tool for size preservation of large and complex shaped tissue-engineered constructs.

Although in this study only lobular-type microtia patients were included, this method can potentially be applied to all types of auricular deformation. Scanning plaster models casted from the patient relieves the diagnostic burden on the patient and obviates radiation exposure. In the future, handheld 3D laser scanners may make the process even easier [57]. The presence of small bubbles in the plaster and cotton wads in the ear canal are of little importance in the measurements, as these may only influence the results at square millimeter level. One limitation of the current study is the use of two different types of CT scanners and the subjective determination of the boundaries of the base of the ear. Nevertheless, the difference in resolution between the two scanners is only marginal, and the potential fluctuations arising from the above factors are on a negligible square millimeter scope.

CONCLUSION

This retrospective study is one of two studies looking at the area of skin covering the auricle. It determined the exact skin surface area of the healthy human auricle and proposed a new method by which accurate calculation of the skin deficit in microtia patients can be achieved. This method could aid reconstructive surgery in clinical practice. Our study demonstrates that microtia patients have a deficiency of more than 50 % when compared to the healthy ear. Supplementing this amount of skin one way or another in microtia patients may improve healing after auricular reconstruction and diminish excessive forces within neocartilage development in engineered constructs. Future studies should be performed to evaluate the use of this method to analyze aesthetic results after ear reconstruction or the usage during clinical practice, *e.g.* to determine the size of the skin graft during the second stage of ear reconstruction.

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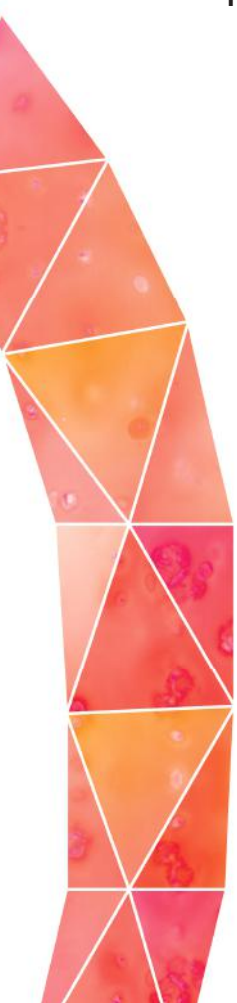
CHAPTER 6

BIOFABRICATION OF A SHAPE-STABLE AURICULAR STRUCTURE FOR THE RECONSTRUCTION OF EAR DEFORMITIES



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ABSTRACT

Bioengineering of the human auricle remains a significant challenge, where the complex and unique shape, the generation of high-quality neocartilage, and shape preservation are key factors. Future RM-based approaches for auricular cartilage reconstruction may benefit from a smart combination of various strategies. Our approach encompassed the fabrication of a hybrid ear-shaped construct using bioprinting techniques, a recently identified progenitor cell population, previously validated biomaterials and smart scaffold design. Cellular performance after extrusion printing of human auricular cartilage progenitor cells (AuCPC) was assessed. Also, compressive properties of 3D-printed poly-ε-caprolactone (PCL) scaffolds were evaluated. Through combining these fiber network structures with cell-laden gelatin methacryloyl (gelMA) bioinks, hybrid auricular frameworks were generated, which were cultured *in vitro* in chondrogenic media for 30 days. The cellular performance of auricular cartilage progenitor cells was unaffected by the printing process. Reinforcing scaffolds increased the compressive properties of the gelMA hydrogel to similar ranges as the native auricular cartilage. Biofabricated hybrid auricular structures exhibited excellent shape fidelity compared to the 3D digital model and displayed the deposition of cartilage-like matrix in both peripheral and central areas of the auricular structure. This study demonstrated the successful fabrication of shape-stable hybrid auricular cartilage constructs, using novel cartilage progenitor cells, mechanical reinforcement, and an anatomically enhanced auricular framework. Our strategy ensured adequate preservation of the auricular shape during a dynamic *in vitro* culture period and enabled chondrogenically-potent progenitor cells to produce abundant cartilage-like matrix throughout the auricular construct. The combination of smart scaffold design with 3D bioprinting and cartilage progenitor cells holds promise for the development of clinically translatable regenerative medicine strategies for auricular reconstruction.

BACKGROUND

Regenerative medicine (RM) is a promising future treatment option for auricular cartilage damage and congenital deformations [25, 65, 159]. It typically applies a combination of cells, materials and bioactive factors to engineer a new tissue [187]. As current surgical strategies for auricular reconstruction utilize autologous costal cartilage for shaping the implant framework [39, 105, 235, 239, 335], the generation of neocartilage in the laboratory would obviate the need for a large harvest site and thus reduce associated morbidity [25, 65, 159, 350, 367]. In addition, RM has the potential to further mimic the structural and constitutional complexity of native tissue [159, 391]. Compared to the rigid costal cartilage framework [25, 159] or the synthetic alternative MedPor® [298], the tissue-engineered auricular implant should ideally exhibit biochemical and mechanical properties that are more similar to the native elastic cartilage [159, 257]. The first clinical trial with tissue-engineered ear-shaped constructs implanted in five children presents encouraging preliminary outcomes [388].

Nevertheless, tissue-engineered auricular constructs still face a number of challenges before they become viable alternatives for currently applied reconstructive strategies. The human auricle presents a complex structure that is difficult to produce and maintain. Firstly, its unique shape requires a patient-specific approach while highlighting the anatomical details in order to ensure an aesthetically satisfactory result after implantation under the cranial skin [17, 266]. Secondly, the maintenance of that shape should be ensured for a lifetime, requiring excellent cellular performance and a long-term balance between stiffness and flexibility. This means that cartilage matrix deposition should be abundant and appropriately organized to properly mimic the native tissue's microscopic anatomy and biomechanical properties. However, especially during the first stages of tissue development and maturation these properties are inferior to the native situation, and deformation and collapse are frequently reported in longer-term *in vivo* studies evaluating tissue engineered ear-shaped constructs [25, 66, 315]. Necrosis due to nutrient limitation and an inferior mechanical integrity of the developing neo-tissue may be contributing factors [32, 66, 257, 266]. Thirdly, the production of a large structure requires a significant number of autologous cells. Native chondrocytes lose their chondrogenic phenotype upon repeated expansion [144, 288, 303, 307] and then produce a more fibrocartilage-like matrix. Mesenchymal stromal cells are readily expandable but may favor hypertrophic differentiation and the endochondral ossification pathway, resulting in a mineralized matrix that may lead to rigidity and implant extrusion [120, 229]. Thus, RM approaches for auricular cartilage reconstruction would benefit from customized patient-specific shapes with adequate reinforcement and improved tissue quality before translation to daily clinical practice would be suitable.

Biofabrication-based RM uses additive manufacturing technology with cells and supporting materials as building blocks to create living structures [132], with the goal to recapitulate and restore functions of native tissues [191]. Through a computer-aided design and manufacturing (CAD/CAM) process, patient-specific and customizable shapes can be generated [164, 170, 295, 391]. The technology's ability to deposit multiple materials with high control over the structural organization allows the fabrication of complex external and internal architectures [266]. Reinforcing scaffolds can be combined with cell-laden hydrogels to create hybrid constructs with improved performance on a biochemical and biomechanical level compared to traditional tissue engineering strategies [278, 364, 369]. As an alternative to chondrocytes or mesenchymal stem cells, cartilage progenitor cells could be used. These cells can be harvested through a small biopsy from the patient's normal external ear or cartilage remnants on the affected side and can be expanded to high cell numbers while maintaining a potent chondrogenic differentiation capacity [265, 368]. The way towards clinical application of engineered cartilage may well be a smart combination of various strategies.

In view of this, our study combined smart scaffold design with a progenitor cell population for the biofabrication of an auricular cartilage structure. For the first time, a population of novel human auricular cartilage progenitor cells (AuCPC) was applied in bioprinting and for the fabrication of an ear-shaped construct. We evaluated cellular performance after the printing process and determined an appropriate reinforcing scaffold design to support the biomechanical properties of the developing cartilage. The auricular shape was designed to match current surgical strategies to enhance the native anatomical details. Dual-printed auricular constructs were cultured *in vitro* and assessed for shape fidelity and biochemical composition. The fabrication of a mechanically reinforced and anatomically enhanced structure in combination with chondrogenically potent AuCPCs provides an interesting avenue for the development of clinically translatable cartilage RM strategies.

MATERIALS & METHODS

Isolation of cartilage progenitor cells

Auricular cartilage progenitor cells (AuCPC) were isolated from fresh human auricular cartilage. The auricles of recently deceased elderly donors (AuCPC-adult; n = 4, mean age 87.5 ± 12.3, range 69-94 years) who had donated their bodies to science were kindly provided by the Department of Anatomy at the University Medical Center Utrecht (The Netherlands). Remnant tissue from pediatric patients undergoing otoplasty (AuCPC-pediatric; n = 3, mean age 7.7 ± 2.1, range 6-10 years) and microtia reconstruction (AuCPC-microtia; n = 3, mean age 10 ± 3.6, range 7-14 years) were provided by the Department of Plastic, Reconstructive & Hand Surgery at the Wilhelmina

Children's Hospital (Utrecht, The Netherlands). Anonymization was performed and tissues were obtained in accordance with the ethical guidelines of the University Medical Center Utrecht.

AuCPCs were isolated from the minced cartilage as previously described [265]. Briefly, enzymatic digestion was applied using a 0.2 % pronase (Roche, USA) solution for 2 hours followed by a 0.075 % collagenase type II (Worthington Chemical Corporation, USA) digestion for 16 hours at 37 °C. After filtration through a 100 µm cell strainer and centrifugation for 5 minutes at 300 ×*g*, pelleted cells were resuspended in Dulbecco's modified Eagle Medium (DMEM; 31966, Gibco, The Netherlands) and plated at a density of 500 cells/cm^2 in fibronectin-coated culture flasks and incubated for 20 minutes at 37 °C. Non-adherent cells were removed and the remaining attached cells were cultured in chondroprogenitor expansion media, consisting of DMEM supplemented with 10 % v/v fetal bovine serum (FBS; Lonza), 0.2 mM L-ascorbic acid 2-phosphate (Sigma-Aldrich, The Netherlands), 100 U/mL penicillin (Life Technologies, The Netherlands), 100 µg/mL streptomycin (Life Technologies) and 5 ng/mL basic fibroblast growth factor (bFGF; Peprotech, UK). After expansion, cells were stored in liquid nitrogen until further use.

Preparation of cast and printed cell-laden hydrogel samples

The impact of extrusion printing on AuCPCs was assessed by performing viability, metabolic and biochemical assays. For this, AuCPCs were encapsulated in gelMA hydrogel either with or without prior extrusion.

Gelatin methacryloyl (gelMA) was synthesized according to a previously published protocol [218]. Briefly, gelatin type A (obtained from porcine skin; Sigma-Aldrich) in PBS was functionalized with methacrylic anhydride groups to an 80 % degree of functionalization. In order to obtain a hydrogel, a 10 % w/v solution of gelMA was supplemented with 0.1 % w/v 2-hydroxy-1-[4-(2-hydroxyethoxy) phenyl]-2-methyl-1-propanone (Irgacure 2959; BASF, Germany) as a photoinitiator.

AuCPCs were expanded to passage 4 and were suspended in gelMA at a density of 1.5×10^7 cells/mL. For the cast group, cylindrical constructs (diameter = 6 cm, height = 2 mm) were generated by casting the cell-laden hydrogel into a custom-made Teflon™ mold and subsequently applying UV-radiation for 5 minutes (wavelength $\lambda = 365$ nm, intensity E = 3 mW/ cm^2, at height of 2 cm; 144 portable UV lamp, Vilber Lourmat, Germany) to elicit free-radical polymerization. For the printed group, the cell-laden hydrogel was first extruded through a microvalve (CF300, MVC03-006; RegenHU, Switzerland) using a multi-material bioprinting device (3D Discovery DD 135N, RegenHU) at 37 °C with a pressure of 0.05 MPa and a valve opening time of 400 µs, and then resuspended and cast into the mold following the above protocol. Control samples were constructed for both groups, following the same procedures with a cell-free hydrogel.

Samples were cultured at 37 °C and 5 % CO_2 in chondroprogenitor differentiation medium, consisting of DMEM supplemented with 1 % v/v insulin- transferrin-selenous acid (ITS+ Premix; Corning, USA), 0.2 mM L-ascorbic acid 2-phosphate (Sigma-Aldrich), 100 U/mL penicillin (Life Technologies), 100 µg/mL streptomycin (Life Technologies), 100 nM dexamethasone (Sigma-Aldrich) and 10 ng/mL transforming growth factor β1 (TGF-β1; Peprotech). Culture medium was refreshed every 3 days.

Assessment of cell viability in cast and printed samples

The effect of extrusion printing on the viability of AuCPCs was assessed using a LIVE/DEAD cell viability assay. On days 1, 3 and 10 of culture, hydrogel samples from the cast and printed group (16-19 replicates per condition) were incubated in 0.1 % calcein-AM and 0.1 % ethidium homodimer-1 (Life Technologies, USA) for 20 minutes at room temperature. Cells were visualized using a confocal microscope (Leica SP8 X, USA), displaying a green and red color for live and dead cells respectively. Live and dead cells were counted, and cell viability was calculated using the following formula:

$$\text{Cell viability} = \frac{\text{Live cells}}{\text{Live+dead cells}} \times 100\%$$

Evaluation of metabolic activity in cast in printed samples

Metabolic activity as an indicator of cellular health was evaluated using a resazurin assay on days 1, 3 and 10. A 440 mM stock solution of resazurin (Alfa Aesar, Germany) was diluted with differentiation medium in a 1:10 ratio. Subsequently, hydrogel samples from the cast and printed group (24 replicates per condition) were incubated in this solution in the dark at 37 °C. After 4 hours incubation, fluorescence of resorufin (reduced from the resazurin agent) was measured at 544 nm excitation and 570 nm emission using a spectrofluorometer (Fluoroskan Ascent FL; ThermoFisher, USA). The resulting fluorescence is reported here, after correction for blanks.

Quantification of glycosaminoglycans in cast and printed samples

At day 28 of culture, 16 replicates of each group were collected and prepared for biochemical evaluation. Lyophilized samples were digested overnight at 60 °C in 250 µL papain digestion buffer (P3125; Sigma-Aldrich), consisting of 0.2 M NaH_2PO_4 (Merck, USA) and 0.01 M ethylenediaminetetraacetic acid (EDTA; VWR, USA) in milliQ water (pH = 6.0), supplemented with 250 µL/mL papain solution (48 units/mg of protein; Sigma-Aldrich) and 0.01 M cysteine (C9768; Sigma-Aldrich).

Total double-stranded DNA (dsDNA) content was quantified using a Quant-iT PicoGreen dsDNA assay (Life Technologies). A spectrofluorometer (Fluoroskan Ascent FL; ThermoFisher) was used

to measure fluorescence at 485 nm excitation and 520 nm emission. Results were corrected for the dilution factor and compared to a standard of known concentrations of DNA.

Glycosaminoglycans were quantified as a measure of cartilage-specific matrix production following a dimethylmethyleneblue (DMMB; Sigma-Aldrich; pH = 3.0) assay. The 525/595 nm absorbance ratio of the reagent was determined with a VersaMax plate reader (Molecular Devices, UK). Taken into account the dilution factor, a standard of known concentrations of chondroitin sulphate C was used to calculate the content of sulphated GAG (sGAG).

sGAG and dsDNA content in each sample were both normalized against the dry weight of the sample. The ratio of sGAG per dsDNA was calculated to display the cartilage-specific matrix-production activity of single cells in the hydrogel.

Preparation of supporting PCL scaffolds with various strand spacings

Properties of supporting scaffolds were evaluated through mechanical and biochemical assessment. For this, printed scaffolds with various fiber spacings were fabricated and combined with cell-free or cell-laden hydrogel. Supporting scaffolds were printed through Fused Deposition Modeling (FDM) using the 3D Discovery bioprinter. Fabrication occurred in a layer-by-layer manner to create a woodlog 0°-90° organization with strand spacings of 400 µm, 800 µm, 1000 µm and 1200 µm. Medical grade poly-ε-caprolactone (PCL) (Purasorb PC 12, Lot# 1412000249 03/2015; Corbion Inc., The Netherlands) was heated to 80 °C in the printhead and extruded through a 27 G needle (Integrated Dispensing Solutions, USA) at a pressure of 0.6 MPa with a feed rate of 0.7 mm/s, resulting in a strand thickness of 300 µm. Scaffolds were first printed in 60 mm (L) x 10 mm (W) x 2 mm (H) sheets, after which cylindrical samples were obtained by applying a biopsy punch (diameter = 5 mm; BAP Medical, The Netherlands).

Compression testing on supporting scaffolds

In order to evaluate compressive properties of printed PCL scaffolds of the varying strand spacings, both PCL scaffold-only and hybrid hydrogel-PCL samples (5 replicates per condition) were subjected to unconfined uniaxial compression testing. For the hybrid group, scaffolds were inserted into the previously described Teflon™ mold and injected with a photoinitiator-supplemented 10 % w/v gelMA hydrogel, followed by UV-crosslinking as described above. Hybrid samples were submerged in PBS for 24 hours before testing. Tests were performed on a Z010 mechanical tester (Zwick Roell Kennesaw, USA) using a loadcell of 1 kN and a compression speed of 1 mm/min. The compression modulus was obtained by calculating the slope of the stress/strain curve in the 10 % to 15 % strain range.

Biochemical evaluation of cell-laden hybrid scaffolds

Hybrid scaffolds were prepared in the same manner as described above, yet with a 10 % w/v hydrogel laden with AuCPCs at a density 1.5×10^7 cells/mL. Cell-laden gels without supporting PCL scaffolds were fabricated as controls. Samples were cultured in chondroprogenitor differentiation medium at 37 °C and 5 % CO_2. Medium was refreshed 3 times per week. After 28 days of culture, samples were harvested and prepared for biochemical testing. Quantification of GAGs and DNA was performed as previously described.

Hybrid bioprinting of auricular constructs and chondrogenic differentiation in dynamic culture

As a proof-of-principle, an auricular construct was fabricated that combined the printing of human AuCPCs with a reinforcing PCL scaffold. After 30 days of *in vitro* pre-culture, the printed ears were assessed on shape fidelity and biochemical composition by means of µCT scanning and processing, biochemical quantification, histology and immunohistochemistry.

A modular auricular implant was designed that aligned with current surgical strategy and resulted in a satisfactory aesthetic appearance, as reported previously [266]. The base module of this design was used for this proof-of-principle experiment, with dimensions of 38.9 (L) x 25.35 (W) x 2.0 (H) mm. The 3D ear model was transcribed into the corresponding G code for the 3D Discovery bioprinter. Using the previously reported specifications, contoured PCL scaffolds were printed with a strand spacing of 1000 µm.

AuCPCs were expanded up to passage 4 and encapsulated in gelMA at a density of 1.5×10^7 cells/mL. The cell-laden hydrogel was extruded through the microvalve into the ear-shaped scaffold. The hybrid constructs were immediately photocrosslinked by UV-radiation for 15 minutes (wavelength $\lambda = 365$ nm, intensity $E = 7$ mW/cm^2, at a height of 12 cm; CL-1000L UV Crosslinker, UVP, UK).

Printed ear constructs were cultured at 37 °C and 5 % CO_2 in chondroprogenitor differentiation medium for 30 days in a bioreactor with a stirring rate of 18 rpm. Media was refreshed twice per week.

Assessment of distribution of glycosaminoglycans through contrast-enhanced micro-computed tomography and histology

In order to assess the overall geometry of the printed constructs, as well as the 3D distribution of neo-synthesized cartilage matrix, printed ear samples were harvested at day 1 and day 30 (3 replicates per timepoint) and incubated in a PBS solution containing 12 mg/mL of CA4+ (MW = 1354 g/mol, q = +4) for 4 hours at 37 °C. The cationic contrast agent CA4+, exhibiting high affinity for negatively charged glycosaminoglycans [115], was synthesized as previously described [325]. After

incubation, the samples were removed from the contrast agent solution and scanned with a micro-computed tomography scanner (μCT; Quantum FX, Perkin Elmer, USA). For X-ray attenuation measurements, this occurred at a voxel size of 60 μm^3 with 70 kV tube voltage and 200 μA tube current for 17 seconds. For comparison with histology, voxel size was 50 μm^3, tube voltage was 70 kV, and tube current was 200 μA for 4.30 minutes. 3D reconstruction was carried out automatically after completion of each scan using the scanner's software (Quantum FX μCT software, Perkin Elmer).

Image analysis was performed using Fiji (software version 1.50; National Institutes of Health, USA). Mean X-ray attenuation values on the Hounsfield scale were obtained by averaging attenuation values over all the coronal μCT slides. Constructs for histology were fixed in 4 % neutral buffered formalin, dehydrated through a graded ethanol series, cleared in xylene and embedded in paraffin. Constructs were sectioned into slices of 5 μm thickness in the same direction and orientation as the μCT slices. The deposition of cartilage glycosaminoglycans was evaluated through a triple stain consisting of hematoxylin, Fast Green and Safranin O. Collagens were visualized through immunohistochemistry as previously reported [266], with appropriate antibodies for collagen type II (II-II6B3; DSHB, USA) and collagen type I (ab138492, 1:400; Abcam, UK). All sections were mounted in DPX mounting media (Millipore, USA) and examined using a light microscope (Olympus BX51; Olympus, Germany). Histological images were compared to their corresponding slices in the μCT stacks using Fiji.

Determination of shape and size retainment of printed constructs

Retainment of shape of the printed ears was evaluated by comparing the 3D images obtained by μCT at day 1 and day 30 to the original digital design. The μCT images (pixel dimensions 0.118 x 0.118 x 0.118 mm) were segmented and converted to a dense 3D surface mesh model using 3D Slicer [96]. Incomplete hydrogel filling of the scaffold was corrected by manual closure of the holes in order to accommodate a clean comparison of scaffold shapes. Shape comparison requires dense models with evenly distributed 3D points; hence, all models including the original digital design were remeshed in Meshmixer (Autodesk, USA). Subsequently, the printed ear models were compared with the original design. First, both models were aligned in the coordinate system through the Iterative Closest Point Algorithm (ICP) using the alignment tool in MeshLab (Visual Computing Lab, Italy). Then, the distance of each 3D point in the printed ear model to its closest corresponding 3D point in the original design model was calculated using the Hausdorff distance filter in MeshLab. The minimal, maximal, mean and root mean square (RMS) distance of each comparison were determined and the distances were visualized in a 3D color map. A margin of 1.5 mm was determined as an acceptable maximum deviation from the original digital design [388].

Statistical analysis

Quantitative results are expressed as mean ± standard error of the mean (SEM). Comparisons between cast and printed cells at different timepoints were performed through a two-way ANOVA. Results of mechanical properties of scaffolds were assessed using a two-way ANOVA, whereas biochemical composition between the various strand spacings was evaluated using an ordinary one-way ANOVA. Bonferroni post-hoc tests were applied to these analyses. Quantitative results from the printed ears were analyzed with an unpaired t-test. Statistical analyses were carried out using Graphpad Prism 8 (Graphpad Software, USA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Extrusion printing does not negatively affect cell viability, metabolic activity and GAG production

LIVE/DEAD staining was performed on AuCPCs in gelMA hydrogel at days 1, 3 and 10. Both cast and printed groups displayed predominantly green stained cells throughout the samples at all timepoints (Figure 1A). Cells were distributed homogeneously throughout the gel. Quantification of live and dead cells confirmed high viability rates in both cast and printed groups (Figure 1B). Cast constructs displayed 98.62 % ± 0.43, 98.82 % ± 0.56 and 99.12 % ± 0.3 live cells at day 1, day 3 and day 10, respectively. Cells within printed constructs performed similarly, with a viability rate of 97.98 % ± 0.81, 98.72 % ± 0.52 and 98.10 % ± 0.73 at days 1, 3 and 10. No significant differences were observed between cast and printed groups and over time.

Metabolic activity as measured through fluorescence of resorufin (Figure 1C) was 69.85 ± 4.61, 81.29 ± 2.72 and 75.45 ± 5.45 arbitrary units (AU) at days 1, 3 and 10 in the cast group. Values of printed constructs were comparable: 68.04 ± 2.91, 79.99 ± 1.63 and 73.53 ± 4.62 at days 1, 3 and 10, and no significant differences were observed between groups.

Production of GAGs was assessed after 28 days of *in vitro* chondrogenic culture (Figure 1D). No significant differences in GAG production were observed between groups. The mean GAG per DNA of cast samples was 28.17 ± 4.65 µg/µg and for printed samples this was 23.41 ± 4.20 µg/µg. Nevertheless, there were notable differences between individual donors in each separate donor group (adult, pediatric and microtia), where for some donors only little matrix synthesis was observed. AuCPCs from adult donors produced a mean GAG per DNA of 32.42 ± 6.59 µg/µg when cast and 28.51 ± 8.21 µg/µg when the cells were printed. Cells from pediatric auricular cartilage produced 31.05 ± 14.60 µg/µg in cast constructs, whereas cells in printed samples produced 24.60 ± 10.68 µg/µg. Constructs with microtia-derived AuCPCs contained 22.00 ± 5.62 µg/µg GAG per DNA when cast and 23.41 ± 4.20 µg/µg when printed.

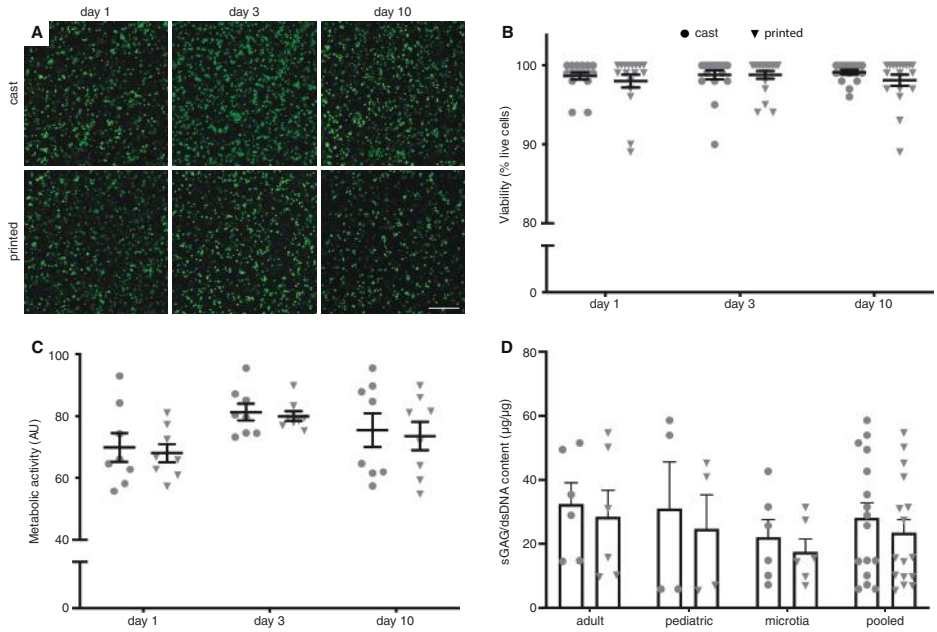

Figure 1. Effects of extrusion printing on viability, metabolic activity and chondrogenic differentiation of AuCPCs. LIVE/DEAD staining on cast and printed constructs after 1, 3 and 10 days of culture (A). Representative images of one donor are shown, where green indicates live cells and red indicates dead cells. Quantification of live cells as a percentage of the total counted cells at the different timepoints (B). Fluorescence of resorufin as an indication of metabolic activity at days 1, 3 and 10 (C). Production of cartilage-specific proteoglycans by quantification of glycosaminoglycans after 28 days in chondrogenic culture (D). Scale bar in panel A equals 250 µm. No significant differences ($p < 0.05$) between cast and printed conditions were observed.

PCL scaffolds with various strand spacings allow for glycosaminoglycan production

FDM-printed PCL scaffolds were fabricated with strand spacings of 400 µm, 800 µm, 1000 µm and 1200 µm. Compressive properties of empty scaffolds and hybrid scaffolds with 10 % gelMA were determined (Figure 2A). Scaffolds of 400 µm without gel exhibited a compressive Young's modulus of 18.0 ± 0.9 MPa; significantly higher than 5.9 ± 0.1 MPa, 4.1 ± 0.2 MPa and 4.4 ± 0.3 MPa in 800 µm, 1000 µm and 1200 µm scaffolds, respectively. Incorporating 10 % w/v gelMA into the 800 µm and 1000 µm scaffolds significantly increased the compressive properties to 12.2 ± 1.1 MPa and 10.6 ± 1.9 MPa respectively. The effect of incorporating reinforcing fiber structures on the production of GAGs was assessed by comparing hybrid constructs with non-reinforced, hydrogel-only samples (Figure 2B). No significant effect of incorporating PCL fibers was observed on the ability of AuCPCs to produce GAGs.

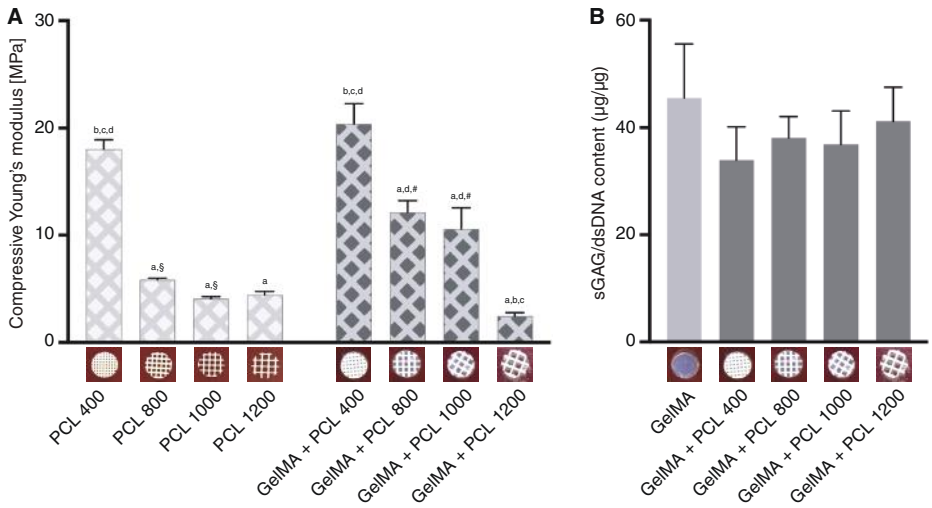

Figure 2. Scaffolds with varying strand spacings exhibit different compressive properties but display similar GAG production. Compressive Young's modulus in PCL scaffolds and hybrid gelMA + PCL scaffolds with strand spacings of 400, 800, 1000 and 1200 µm (A). Production of glycosaminoglycans in hydrogel and hybrid gelMA + PCL samples, as normalized to dsDNA content (B). A significant difference in compressive modulus to the 400 µm condition is indicated by [a], to the 800 µm condition by [b], to the 1000 µm condition by [c] and to the 1200 µm condition by [d]. § indicates a significant difference from the PCL-only condition to the hybrid condition, and # signifies a significant difference from the hybrid condition to the PCL-only condition. No significant differences in glycosaminoglycan production were observed.

Straight non-sagging fibers could be produced using the FDM printing technique. Fabricated scaffolds demonstrated excellent top (Figure 3A) and side (Figure 3B) porosity. The digital 3D model of the auricular structure (Figure 3C) could reliably be fabricated with a strand spacing of 1000 µm while maintaining fiber quality and porosity (Figure 3D). Cell-laden gelMA hydrogel was homogenously distributed throughout the auricular scaffold (Figure 3E), with a few local exceptions where gel had leaked out after printing. During 30 days of dynamic *in vitro* culture, the hybrid auricular structures remained intact (Figure 3F).

Excellent shape fidelity of printed constructs directly after printing and after 30 days *in vitro*.

Shape accuracy after printing and retainment in *in vitro* culture was determined through µCT scanning (Figure 4A and 4C) and subsequent computing of the distance between closest corresponding 3D points (Figure 4B and 4D). The day after printing, the fabricated auricular shape corresponded to the original digital design with a mean deformation of 0.13 mm (min = 0 mm, max = 1.80 mm, RMS = 0.19 mm). After 30 days in culture, average deviation from the digital model increased to 0.21 mm (min = 0 mm, max = 1.47 mm, RMS = 0.28 mm). This increase of 0.08 mm is

less than the spatial resolution of the µCT (voxel size = 0.118). Both distance color maps display an array of predominantly red, orange and yellow colors, indicating distances of <0.5 mm.

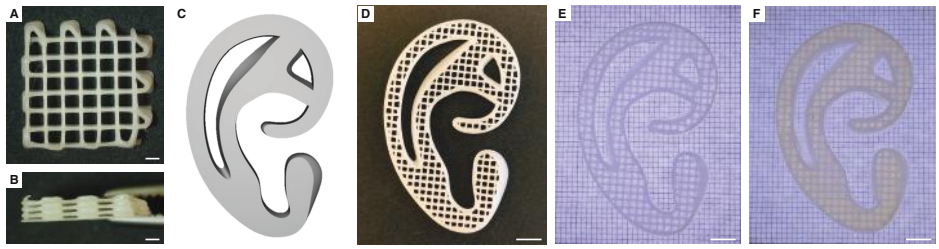

Figure 3. Fabrication of the PCL-based reinforcing auricular scaffolds. Top (A) and side (B) view of scaffolds with 1000 µm strand spacing. Digital 3D model of auricular module (C). PCL scaffold of the auricular module (D). Hybrid cell-laden gelMA-PCL auricular module after printing (E) and after 30 days of *in vitro* culture (F). Scale bars equal 1000 µm in panels A/B and 5 mm in panels D/E/F.

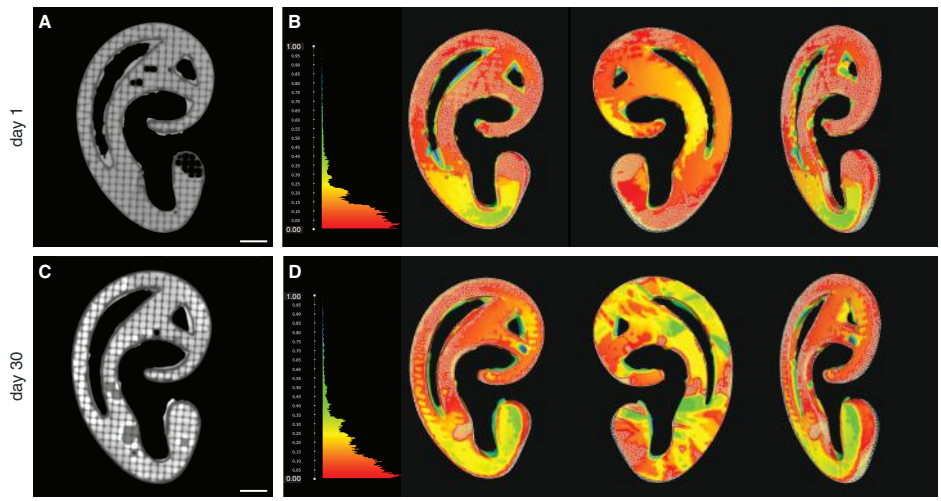

Figure 4. Assessment of shape fidelity of printed auricular constructs. µCT images of ear-shaped constructs in coronal plane after 1 (A) and 30 (C) days of *in vitro* pre-culture. Scale bars equal 5 mm. Shape conformity of printed shapes compared to the original digital design after dynamic culture of 1 day (B) and 30 days (D). The Hausdorff distances from each point in the printed shape to the closest point in the digital model are visualized in a color distance map, ranging from red (0.00 mm) to blue (1.00 mm). The digital design is visualized as a triangle mesh.

Cartilage-specific components are produced abundantly throughout the auricular construct

The distribution of GAGs within printed cell-laden auricular constructs was determined by contrast-enhanced μCT and histological evaluation. Compared to day 1 (Figure 5A), auricular constructs cultured for 30 days displayed increased GAG content (Figure 5B). X-ray attenuation values increased significantly over the 30-day culture period, with a mean of 547.7 ± 35.9 HU at day 1 and 913.2 ± 51.4 HU at day 30 (Figure 6A). GAGs were distributed throughout the auricular module, with qualitatively higher intensities in the helix, antihelix and tragus areas (Supplementary Video 1). Production and distribution of GAGs was confirmed through Safranin O staining of a matching slice (Figure 5C). There was abundant pericellular labelling gradually dispersing into the inter-territorial matrices (Figure 5D). GAG production was confirmed through biochemical analysis, which showed a significant increase compared to day 1 (Figure 6B). Mean GAG per DNA was 17.62 ± 0.32 μg/μg at day 30 compared to 0.74 ± 0.16 μg/μg at day 1. Collagen type II displayed a similar organization as glycosaminoglycans, with intense intracellular brown staining with a gradual distribution into a wide inter-territorial organization (Figure 5E). Collagen type I staining was of less intensity and localized mainly in the pericellular areas (Figure 5F).

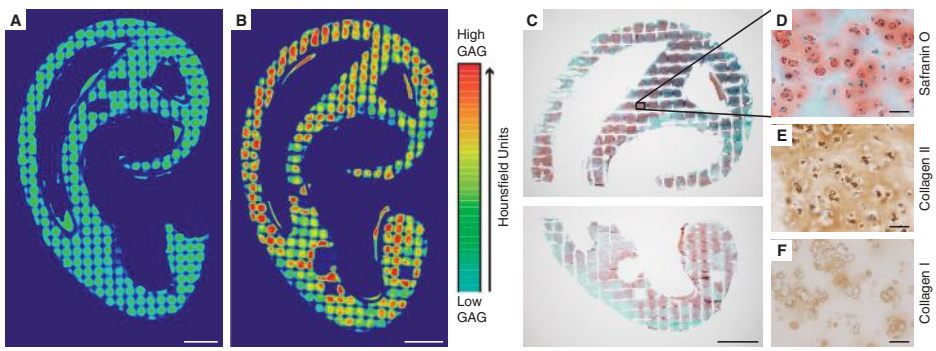

Figure 5. Qualitative analysis of deposition of cartilage matrix components in bioprinted auricular constructs after 30 days of in vitro culture. X-ray attenuation obtained by μCT scanning after CA4+ incubation at day 1 (A) and day 30 (B), as expressed in Hounsfield Units. Safranin O staining visualizing the distribution of glycosaminoglycans throughout the auricular sample (C) and at 10X magnification (D). Immunohistochemistry visualizing the deposition of collagen type II (E) and collagen type I (F). Scale bars in panels A/B/C equal 5 mm and in panels D/E/F equal 100 μm.

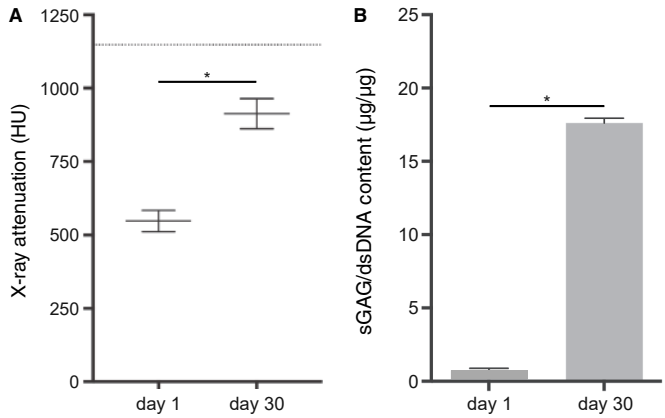

Figure 6. Quantitative analysis of glycosaminoglycan production of bioprinted auricular constructs. Mean X-ray attenuation values on the Hounsfield scale (A) and mean GAG per DNA (B) after 30 days of in vitro culture. The mean value from a sample of native pediatric auricular cartilage (1 donor, 2 replicates; 1147.6 HU) is included as a reference. The asterisk (*) indicates a significant difference ($p < 0.05$) between timepoints.

DISCUSSION

RM-based treatment of auricular deformities has the potential to surpass clinical outcomes of the current gold standard reconstructive techniques. Nevertheless, the intricate three-dimensional shape of the auricle, the biochemical composition of the auricular cartilage and its biomechanical properties present ongoing challenges in the generation of auricular implants. Several promising strategies have been reported in terms of patient-specific implant design [14, 54, 61, 164, 201, 295], cartilage-like tissue regeneration [265, 291, 382, 387], construct reinforcement [217, 363] and hybrid bioprinting [125, 165, 170]. Digital photogrammetry and CAD/CAM technology can aid in the creation of patient-specific auricular shapes [33, 67, 164, 201, 295] that can be fabricated with improved internal spatial organization using biofabrication techniques. Kim *et al.* (2019) 3D printed porous polyurethane auricular scaffolds that demonstrated improved cartilage ingrowth in comparison to commercially available MedPor® implants [173]. In the study by Zopf *et al.* (2014), 3D-printed patient-specific auricular scaffolds seeded with chondrocytes were implanted subcutaneously in mice and showed evidence of shape maintenance and neocartilage matrix deposition *in vivo* [391]. Kang *et al.* (2016) presented an integrated tissue-organ printer with which reinforced hybrid auricular constructs were fabricated that displayed cartilage matrix production after 2 months *in vivo* [170]. Tissue-organ printing systems have also been successful in the fabrication of multi-tissue auricular constructs, as exemplified by the studies by Lee *et al.* (2014) [190] and Jung *et al.* (2016) [165] where both the auricular cartilage and the fatty earlobe were printed in a composite construct. Despite these collective efforts, the 'bioengineered auricle' is

not yet ready for widespread clinical application. Indeed, only one study has thus far described the application of tissue-engineered auricular constructs in patients, with encouraging results. Zhou *et al.* (2018) 3D printed a patient-specific mold in which autologous microtia chondrocytes were seeded onto a composite polymer scaffold and implanted in 5 children after a 12-week *in vitro* pre-culture period. Obvious cartilage formation was observed prior to implantation and there were no signs of absorption or extrusion during a 2.5-year follow-up [388]. Nevertheless, the histological and aesthetical outcomes do not fully match the composition and complex geometry of the native auricle yet. Patient-specific shapes, adequate construct reinforcement, enhanced cellular function and improved quality of the neo-tissue remain key challenges for clinical translation of engineered auricular structures. Future RM-based approaches for auricular cartilage reconstruction may benefit from a smart combination of various strategies.

Our approach entailed fabricating a hybrid ear-shaped scaffold using bioprinting techniques, a recently identified progenitor cell population, previously validated biomaterials and smart scaffold design. The combination of gelMA and PCL in hybrid constructs has been established as a suitable strategy for RM purposes, evidenced by good outcomes in terms of printability, construct stability, and tissue regeneration in various studies [193, 363, 364]. The hydrogel gelMA fosters chondrogenic differentiation of cartilage progenitor cells [193, 265] and is suitable as a bioink [177], whereas the physicochemical properties of PCL enable additive manufacturing of porous 3D scaffolds that support construct integrity as well as neotissue formation [283, 363, 380]. Results from our study underscore that the incorporation of fiber reinforcement does not affect the ability of embedded cells to produce matrix, as exemplified by the non-significant differences in glycosaminoglycan production in samples with strand distances between 400 µm and 1200 µm. Further, these reinforcing scaffolds enable a necessary increase in compressive properties of hydrogel constructs. In previous studies, we found a maximum compressive Young's modulus of cell-laden gelMA hydrogels of 179.2 kPa after 56 days of *in vitro* culture [265]. Although a significant increase over time was observed compared to the start of culture, these values are insufficient to support the developing neotissue after implantation *in vivo*, where contractive forces of the overlying tissues as well as external forces impact the implanted engineered construct [199, 267]. Reports on mechanical characterization of the native auricular cartilage in humans are limited, and test types and outcome measures vary between studies [130, 255, 390]. Griffin *et al.* (2016) reported the compressive Young's modulus of native human auricular cartilage to range between 1.41 MPa in the helix and 2.08 MPa in the concha of the ear [130]. Clearly, tissue-engineered auricular constructs require structural enhancement in order to sustain the mechanical loading that the construct may experience *in vivo*. This challenge has been recognized and various approaches have been reported in the literature to accomplish mechanical reinforcement. Support of the developing neotissue has initially been provided through external stents [49, 246, 373] or molds [168]. Visscher *et al.* (2019) described a 3D-printed internal porous PCL mold to encapsulate a

cell-laden hydrogel that exhibited compressive moduli between 100-200 MPa depending on porosity [361]. Although this approach will certainly protect the developing neotissue within, this high degree of construct stiffness may cause similar problems to MedPor®, including extrusion and exposure [195]. The incorporation of a metal wire framework in the study by Zhou *et al*. (2011) maintained the dimensions of ear-shaped constructs *in vivo* [389], yet this approach also harbors a high risk of implant extrusion and exposure. The work by Melchels *et al*. (2016) presents a more subtle hydrogel-based reinforcement strategy that enabled tuning of the composite material stiffness between 138 and 263 kPa [217]. A hybrid auricular construct was printed where the cell-laden bioink was reinforced with a strengthening hydrogel. This approach provides a highly cell-friendly environment, yet the compressive properties may still be unable to support the developing neocartilage upon implantation. Fiber reinforcement using biodegradable polymers can further increase biomechanical characteristics of engineered cartilage. PCL has previously been applied for the fabrication of various complex structures [364] and can markedly increase construct stiffness [363]. The studies by Zhou *et al*. (2018), Kang *et al*. (2016), Park *et al*. (2016), Jung *et al*. (2016), Zopf *et al*. (2014) and Lee *et al*. (2014) have all demonstrated the successful 3D fabrication of PCL-reinforced auricular constructs [165, 170, 190, 278, 388, 391]. In our study, reinforcing gelMA samples with FDM-printed PCL fibers increased the compressive modulus to at least 2.49 MPa. This indicates that such fiber-reinforcement could be sufficient to support the engineered auricular construct during tissue maturation *in vivo*, without causing unnecessary rigidity.

A limitation of the current auricular reconstruction strategies is that the implanted material, which is either autologous costal cartilage or synthetic porous polyethylene, is quite stiff and has an unnatural feel [159]. The human auricle is a flexible structure that endures many daily stresses, such as wearing glasses, headphones or a helmet, the rubbing of clothes while dressing, and sleeping or leaning on it. Rigid structures may cause discomfort and pain, and potentially also soft tissue inflammation, skin necrosis and implant exposure or extrusion even after light traumas [73, 110, 159]. The risk of incorporating a stiff fiber network with high compressive properties in the tissue-engineered construct is that the structure can become too rigid. Therefore, it is important to create reinforcement that provides both compressive strength as well as flexibility. Upon handling the scaffolds in this study, the 400 µm and 800 µm scaffolds appeared quite stiff and inflexible, while the 1000 µm and 1200 µm scaffolds allowed an increasing degree of bending upon manual manipulation. However, with increasing strand spacing also comes less control over the fine architecture of the structure. Progress in bioprinting technology could offer new possibilities for improved internal organization. For instance, an interesting option would be to incorporate organized microfibrous 3D PCL meshes fabricated through the Melt Electrowriting (MEW) technique into hybrid constructs. These fibers have been demonstrated to markedly increase the compressive and shear properties of hydrogel-thermoplastic constructs [16, 52, 78, 363] and may allow improved flexibility of engineered auricular constructs without compromising

on other key features. Although this was not tested in our study, it would be advisable for future studies to include 3-point bending tests to assess mechanical characteristics of tissue-engineered auricular constructs [302]. Information on bending behavior of scaffolds would help in designing reinforcing structures with more refined mechanical attributes that can mimic the native situation.

Proper reinforcement of the engineered auricle can offer initial mechanical stability and thus protect implant shape, yet there are also other factors at play impeding the success of the auricular implant. Tissue maturation may be hampered by a limited nutrient supply in avascular constructs [266]. Especially central regions in large constructs may receive too little nutrients for proliferating and differentiating cells to flourish, leading to necrosis and construct deformation [32, 301]. We previously proposed a modular approach in which the full auricular implant is made up of separately fabricated and matured parts that are combined in a later stage [266]. In addition, the design of the auricular shape is based on the current surgical strategy with an open framework, omitting areas in the scapha, fossa triangularis and concha, and emphasizing the natural eminences and depressions of the auricle to take into account the thickness of the overlying skin. This way, anatomical details are preserved when the auricular implant is covered with skin or facial flaps. More importantly, the construct's surface area for diffusion is maximized and diffusion distances for oxygen and other essential nutrients are decreased compared to full-thickness auricular constructs. Qualitative analysis of the distribution of matrix components in our study demonstrated glycosaminoglycan deposition throughout the auricular module, including central areas. Nevertheless, this distribution was non-homogenous and not all areas displayed optimal matrix production after 30 days *in vitro*. An additional strategy, although non-reflective of the native situation, would be to create perfusion channels to allow non-obstructed flow of nutrients into the construct. Kang *et al.* (2016) reported improved cartilaginous matrix formation throughout auricular constructs due to the incorporation of microchannels [170]. Another interesting strategy would be to provide a reservoir of nutrients within the engineered constructs to alleviate metabolic stresses during periods of high nutrient requirement. Armstrong *et al.* (2015) delivered additional oxygen to cells in central areas of large constructs through myoglobin complexes on the cell membrane [7]. Innovative approaches like these can tremendously improve cell survival and tissue development in large engineered constructs like the auricle.

The quality of the neotissue is not only impacted by the nutrient supply, but importantly also by the inherent regenerative potential of the embedded cells. The fabrication of the human auricle requires between 100 and 250 million cells [25, 66] that should be able to generate an organized neotissue that is rich in glycosaminoglycans, collagens and elastin. Cell choice is thus an important factor in the success of the engineered implant, and options that have extensively been explored for cartilage tissue engineering include chondrocytes and mesenchymal stromal cells from various sources. Native chondrocytes are dedicated to chondrogenesis but are also limited

by a dedifferentiation process after repeated expansion, resulting in an inferior fibrocartilage-like matrix [25, 63, 144, 189, 234, 307, 310]. Mesenchymal stromal cells, on the other hand, are readily expandable while maintaining multipotency; however, these cells display a tendency to undergo hypertrophy and differentiate towards an osteogenic lineage [120, 229]. A recent addition to these choices is the subpopulation of progenitor cells residing in the auricular cartilage [265, 385]. These cells can be obtained through a non-deforming biopsy from the auricle and can be expanded to high cell numbers while maintaining a chondrogenic phenotype, and they are able to produce a cartilage-like matrix in gelMA [265]. This study is the first to evaluate cellular performance of human auricular cartilage progenitor cells after a printing process and to apply these cells for the fabrication of a human ear-shaped construct. Our results indicate that extrusion of AuCPCs through a microvalve system does not negatively affect cell viability, metabolic activity and glycosaminoglycan production. Over the course of 10 days, cell viability was at least 98 % and metabolic activity did not differ between cells that were either cast or printed. Similarly, no significant difference was observed in the production of glycosaminoglycans between cast and printed groups after 28 days *in vitro*. Nevertheless, we did observe differences in performance between individual donors. This donor-to-donor variance is a well-known challenge in cells from human sources [327], and would require the characterization of a quality control system to predict the cells' regenerative potential and thus their usability for clinical application. The fabrication and *in vitro* culture of the auricular module was carried out using a well-performing donor from the pool of studied cells. Abundant glycosaminoglycan production throughout the auricular construct was observed after 30 days *in vitro* culture, as visualized through both histological and contrast-enhanced μCT analysis. The latter technique allows for both qualitative and quantitative analysis. Higher HU values signify a higher concentration of the CA4+ contrast agent, which in turn indicates an increase in GAG content. The application of this novel technique allows for the real-time evaluation of GAG production in a non-destructive manner and without hampering chondrogenesis [115]. It provides an opportunity for monitoring engineered auricular constructs pre-implantation. Upon moving tissue engineered constructs towards the clinic, non-destructive and non-disruptive evaluation methods are essential in providing a quality control check before deciding that the tissue engineered construct can be implanted into the patient. This proof-of-concept demonstrates the feasibility of creating hybrid auricular constructs with inherent regenerative potential, as was evidenced by the abundant production of glycosaminoglycans after only 30 days *in vitro* pre-culture.

High shape fidelity of auricular constructs was observed directly after fabrication and was maintained during the *in vitro* pre-culture period. The used printing technique offers a reliable method for the fabrication of an auricular shape with a distinct internal architecture. Only minor deviations from the digital model were observed that were well below a margin of 1.5 mm elected by Zhou *et al.* (2018) [388]. As hydrogel filling of the auricular scaffold was not perfect, manual

correction was applied that influences the results but does not impact deformation outcomes of the scaffold. With an average deviation of 0.21 mm, it can be concluded that shape and size were preserved throughout the dynamic *in vitro* culture period, indicating that the PCL fibers provide adequate construct stabilization during pre-culture prior to implantation.

CONCLUSION

The engineering of auricular cartilage constructs remains challenging due to the lack of regenerative cells and limited mechanical integrity. Hence, a combination of various strategies addressing these issues is likely to improve the development of regenerative tissue products. Although further optimization of the reinforcing scaffold, the printing process, and the culture method may be required, this proof-of-concept study shows encouraging results for the future application of this technology. We demonstrated excellent shape fidelity of the printed auricular constructs during pre-culture, unaffected cellular performance after printing, and abundant cartilage-like matrix deposition throughout the constructs. The fabrication of a mechanically reinforced and anatomically enhanced structure in combination with chondrogenically potent AuCPCs provides an interesting avenue for the development of clinically translatable regenerative strategies for ear reconstruction.

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SUPPLEMENTARY INFORMATION

Supplementary Video S1: 3D representation of glycosaminoglycan distribution in auricular constructs. Distribution of glycosaminoglycans in each segment of the auricular construct after 30 days of *in vitro* culture, visualized by X-ray attenuation as obtained by µCT scanning after CA4+ incubation.

PART III

SOCIETY

BIOFABRICATION RESEARCH IN AN ETHICAL CONTEXT

CHAPTER 7

ETHICAL CONSIDERATIONS IN THE TRANSLATION OF REGENERATIVE BIOFABRICATION TECHNOLOGIES INTO CLINIC AND SOCIETY

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ABSTRACT

Biofabrication technologies have the potential to improve healthcare by providing highly advanced and personalized biomedical products for research, treatment and prevention. As combining emerging techniques and integrating various biological and synthetic components gets increasingly complex, it is important that relevant stakeholders anticipate the translation of biofabricated 3D tissue products into patients and society. Ethics is sometimes regarded as a brake on scientific progress, yet in our perspective, ethics parallel to research does anticipate on societal impacts of emerging technologies and stimulates responsible innovation. For the ethical assessment, the biofabrication field benefits from similarities to the field of regenerative medicine and an increasing ethical awareness in the development of tissue-engineered products. However, the novelty of the technology itself, the increase in attainable structural complexity, and the potential for automation and personalization are distinguishing facets of biofabrication that call for a specific exploration of the ethics in this field. This review aims to highlight important points of existing ethical discussions, as well as to call attention to emerging issues specific to 3D biofabrication, in bench and bedside research and the translation to society.

BACKGROUND

The aim to restore impaired function by repair, replacement or regeneration of cells, tissues or organs, using a combination of converging technologies, is central to the interdisciplinary field of Regenerative Medicine (RM) [75]. One approach herein is the engineering of biological substitutes through the use of living cells, extracellular matrix components, bioactive molecules, and biomaterials [244]. Although promising advances have been achieved in the generation of biological tissue derivatives using traditional tissue engineering approaches, the need to accurately mimic the native architecture of tissue is underscored by our increasing knowledge of the structure/function relationship in both healthy and pathological conditions [244]. Three-dimensional (3D) printing, patterning and assembly techniques allow greatly enhanced control over the spatial positioning of biomaterials. The incorporation of multiple materials into constructs with highly defined external and internal geometries has the potential to achieve increasingly complex structural organizations that are more closely mimicking native tissues in their structure and function [12]. This superior structural organization attainable with 3D biofabrication compared to traditional tissue engineering techniques is believed to improve tissue development, quality and functionality [132].

A main objective of biofabrication for tissue engineering and regenerative medicine is the creation of functional tissues and organs suitable for transplantation, with the ultimate aim to alleviate shortages in tissue grafts and donor organs [269]. However, the output of the technology is not limited to this considerable goal. Tissue-engineered products are also increasingly applied in fundamental research, pharmaceutical drug testing, analysis of biological and chemical agents, and cancer and disease models [230]. Biofabrication machinery can allow automated, cost-effective mass production of tissue-engineered products, whereas the ability to create custom spatial designs and to incorporate autologous derived materials paves the way for highly personalized clinical treatments [70]. A 3D bioprinted ear-shaped implant for auricular reconstruction is an illustrative example of such a personalized treatment [266].

The rapid progress in biofabrication technologies has sparked great enthusiasm and hope for the future of regenerative medicine applications. As combining emerging techniques and integrating various biological and synthetic components gets increasingly complex, it is important that relevant stakeholders anticipate the translation of biofabricated tissue products into patients and society [13]. Especially in translational medicine, dynamic interactions between scientists, clinicians, ethicists, patients, and other members of society are instrumental in enabling effective scientific progress [353]. Hence, a timely exploration of the ethical and societal impacts of biofabrication technologies is essential to promote responsible interdisciplinary innovation. Ethics is sometimes

regarded as a brake on science, yet in our perspective ethics provides moral guidance and the incentive to continuously refocus on the scientific direction and its impact.

For the ethical assessment the biofabrication field benefits from similarities to regenerative medicine and an increasing ethical awareness in the development of tissue-engineered products [348]. However, the novelty of the technology itself, the increase in attainable structural complexity, and the potential for automation and personalization are distinguishing facets that call for a specific exploration of the ethics of biofabrication. This review aims to highlight important points of existing ethical discussions, as well as to call attention to emerging issues specific to 3D biofabrication, in bench and bedside research and the translation to society.

BENCH

In the bench arena, ethical issues revolve predominantly around the use of both animal and human materials. The ethical challenges of biofabrication highlighted in this section, regarding animal experimentation, cell source and biobanking, are similar to those in regenerative medicine.

In regenerative medicine, animals are used as a source of cells, for studying fundamental processes, or as a model to test new innovations [79]. The justifiability of using animals for laboratory experiments is an overarching debate in all biomedical fields, and the use of animals for research is only considered justifiable under strict conditions [100]. Animal studies contribute to a solid base of preclinical data when a relevant animal model is chosen. Nevertheless, the selection of animal models that predict outcomes in humans as closely as possible can still be a challenge [148]. Through the principle of 'modest translational distance', only good animal models contribute to the evidentiary threshold required to move fundamental research into a clinical research phase [87, 88, 175]. These considerations count for all biomedical research, including biofabrication. Although laboratory animals may never become obsolete, there is clear potential to reduce animal experimentation by using *in vitro* tissue models or organs-on-chips as alternatives [8]. Biofabrication can contribute to this goal, as it can rapidly mass produce pieces of tissue for testing, create custom bioreactors, and fabricate chips with intricate architectures.

Stem cells are often key building materials for biofabricated products. Hence, the ongoing debate on the origin, collection, and use of (stem) cells, though common to biofabrication and other biomedical research, is relevant to discuss in this context. Particularly the use of human embryonic and fetal tissues for research has been controversial, though considered morally acceptable under strict conditions in many quarters of the world [323]. Human materials are very valuable for research purposes, and increasingly also for clinical applications. A readily available source of human material is residual tissue, which is obtained during clinical care and would otherwise

be discarded. Biobanking is the organized collection and storage of such biological specimens and their associated information for research purposes [143, 176]. The ethical debate regarding biobanking has largely focused on the appropriate type of consent and privacy. Key here is the realization that, even in the bench phase, the use of human materials necessitates some form of consent, where the donor either gives explicit approval (opt-in) or explicitly objects (opt-out) [143]. Consent can be given for the use of materials for a defined research purpose (specific consent), or for a yet unspecified range of research topics with only a few restrictions based on the donor's preferences (broad consent) [127]. Although in the latter case a specific research question may be absent, a tissue donor can still be informed about the governance structure of the biobank, such as its ethical oversight procedures, privacy policy and information management; this has been posed as 'broad consent for governance' [30]. One important issue in data management is privacy protection, as specimens are usually linked to phenotypic and identifying data. Using anonymous samples is favorable from a privacy perspective, but then clinically relevant unsolicited findings cannot be returned to the donor [35]. Moreover, one could question whether complete anonymization is still possible in this era of genomics research and Big Data [225]. Another issue is whether, if at all, a person retains ownership of the donated tissue, once it is separated from the body [29]. This can become a serious issue once the research has yielded a product that is commercialized, as per example of the immortal HeLa cell line, originating from residual tissue of the unwitting patient Henrietta Lacks [320].

The responsible use of animal and human materials can be justified by social value. This means that the research conducted should add to the body of knowledge that has the potential to improve the wellbeing of patients, individuals in society, or society itself [134]. It is, therefore, good practice for scientists to regularly question what their experiments will lead to, and in what way they can ensure that the results from their research can be exploited in a next step.

BEDSIDE

The translation from bench to bedside requires a timely and thorough ethical reflection, as premature trials could compromise patient safety and damage public perception of the field [353]. Although results from basic research are sometimes moved to the clinic through compassionate care, surgical innovation or even inappropriate use, the clinical trial is the most rigorous approach to evaluate preclinical results in a clinical perspective. First-in-human trials are an exciting and important step in bridging successful bench experiments and bedside application. However, the novelty, complexity and invasiveness of emerging technologies require specific refinement of the standard ethical, legal and regulatory framework of clinical trials [253, 353]. Many of the ethical issues identified for regenerative medicine in previous literature are also applicable to the biofabrication field. Yet, biofabrication adds another layer of complexity by truly converging

emerging technologies, such as stem cell technology and 3D (bio)printing. Whilst every aspect in clinical research ethics deserves consideration, the discussion for biofabrication is especially interesting with regards to balancing risk/benefit, design challenges, and obtaining informed consent.

It is generally agreed that risks to participants of clinical trials must be proportionate to the anticipated benefits to science, society, and/or the individual. Early phases of clinical research are likely to generate more benefits to science than to the participant, while individual risks and burdens are present at all stages [134]. The dynamic interaction between the body and the tissue-engineered product is regarded as the major challenge in determining the possible outcomes [348]. In principle, the regenerative implant becomes integrated with the body and it will be virtually impossible to remove it or reverse its effects [339]. Due to the lack of prior comparators in tissue engineering to base anticipated risks and benefits on, as well as the variability and complexity of the product, the uncertainties and (un)known unknowns are substantial [251]. The known risks of tissue-engineered products are that cells may exhibit tumorigenic potential and that biomaterial interactions may cause undesirable effects. First, there is the risk of transferring pathogens or instigating an immunogenic rejection response to non-autologous cells [79]. Second, after providing the inductive cues, the tissue engineer renders complete control to the implanted cells and the host body. The capacity of stem cells to endlessly self-renew and differentiate into multiple lineages may pose a considerable risk of tumor development, especially since adult cells may already have encountered DNA damage or other detrimental chromosomal or cellular changes precipitating tumorigenesis [51]. In addition, the bioprinting process may harmfully impact the cells through mechanical, thermal and oxidative stresses. Third, the scaffold material used for biofabrication of tissue-engineered products may elicit unwanted effects. Since every item intended for implantation in the human body must comply with certain safety standards, it is essential to evaluate the biocompatibility and safety of biomaterials in the short and long term. Biofabrication of tissue-engineered products demands biomaterials with improved biological functionality, as well as specific printing properties, such as shear thinning and mechanical strength [209]. Since even residues of used reagents can be toxic or can elicit undesirable functional responses in the patient [202], the development of novel biomaterials should regard the presence of potential toxins as well as the interactions between the material, the cells, and the body. Does the novelty and potential of the field grant acceptance of higher risks and more uncertainties? For early trials, it has been suggested to balance risk versus potential value instead of individual benefits [134] As such, benefits of a trial can also include reciprocal value, in which insight is generated into the working mechanism of the evaluated product and the interaction with the body. This type of value is especially important in young innovative fields like biofabrication.

A well-designed randomized controlled clinical trial is generally the most appropriate way to gather robust clinical data. Although adding a control group makes the research scientifically more valid, this is not always practically or ethically possible. The invasiveness of biofabrication applications would require sham procedures in the control groups, which carry inherent risks and burdens and are, therefore, ethically challenging [252]. In some cases of future biofabrication-based applications, such as auricular reconstruction in children, the route of innovative surgery seems more appropriate. It is important to realize that normative considerations may play as an essential role in the study design as scientific validity. In any case, such novel products require complementation of the study with a long follow-up program, since tissue-engineered products have many uncertainties and unknowns, and the long-term effects of biofabricated implants with regenerative potency are especially unknown. Compared to pharmaceutical phase I trials, participant selection in clinical studies with regenerative biofabricated products is also more complex. Since this approach is aimed at restoring damaged, degenerated or diseased tissue, selecting healthy volunteers as trial participants is not appropriate. In comparison to end-stage patients, stable patients with alternative treatment options may experience fewer benefits and higher risks from a novel intervention. However, end-stage patients with no alternative options may be especially vulnerable to therapeutic misconception [339], which is a misunderstanding of patients regarding the purpose of the study [135].

An important imperative in clinical research ethics is that patients make informed decisions about their participation in a clinical study. Hereto, informed consent is essential in avoiding exploitation of vulnerable patient groups and in empowering participants [93]. The many uncertainties of biofabricated products may make it difficult to appropriately disclose information and it may be very challenging to ensure that participants sufficiently understand the risks and benefits. High expectations of the field may cause people to regard biofabrication as the magical solution for difficult medical problems. Especially patients with no alternative treatment options or younger patients with undesirable prognoses (*e.g.* cartilage injury progressing to osteoarthritis) are prone to the therapeutic misconception [251].

SOCIETY

A set of repeating moral patterns of argumentation has been identified that is applicable to any new biomedical technology, where emphasis is often placed on the hard, quantifiable consequences of the technology on the wellbeing of living beings. The debate generally misses explications on the moral changes fostered by technology, such as changes to experience, habits and perceptions, often referred to as soft impacts [260]. Crucial in this discussion is the public's perception of the biofabrication field. Another important societal aspect is the relationship of biofabrication technologies to views on human enhancement.

Emerging technologies and scientific progress generally spark excitement and expectations. The positive portrayal of a new biotechnology – in both media and research proposals – seems increasingly necessary in order to garner attention, attract actors, and secure scarce funding. However, overselling of a product raises societal expectations and nurtures the therapeutic misconception, often leading to public disillusionment as a field fails to deliver. Unrealistic promises can severely damage a field's reputation and the public's trust [259]. The Gartner Hype Cycle [118] visualizes how a technology can go through phases of inflated expectations and subsequent disillusionment before it eventually matures and enters the stage of productivity and application. A relevant illustration of this cycle is the story of tissue engineering, which received widespread attention after the spectacular sight of the 'Vacanti mouse'. In this experiment, engineered cartilage in the shape of the human auricle was subcutaneously implanted on the back of a nude mouse [49] The first successes in tissue engineering indeed sparked hope for the treatment of damaged tissues and failing organs. In their excitement, scientists made bold statements to highlight the potential of tissue engineering, overestimating the possible benefits of an intervention or giving unrealistic timelines for it to reach the clinic in order to attract funding [245]. However, the field could not deliver on its initial promises and the translation was further hampered by the complicated search for appropriate regulations for the Advanced Therapy Medical Product (ATMP) guidelines. As a result, public enthusiasm and trust waned off, as clinical application seemed to be too far away to ever become reality. Presently, biofabrication technology is well on its way up on slope of expectations and is marked as a research field with high potential. What the stories of tissue engineering and other high-potential fields can teach us is that modesty in claims may prevent structural public disappointment and a damaged reputation. Public trust can be earned by presenting concrete steps on the way to the proverbial flag on the hill, the ultimate goal [259]. In this modern day and age, scientific citizenship – the ideal that the public is well informed and able to make decisions regarding scientific research – is becoming increasingly important [124]. A public that is sufficiently aware of the potential impact of a technology on their lives, on a realistic timeline, can provide the researcher with valuable input on the degree of public acceptance, the aspects of the technology people are resistant to, and how a technology can be refined so it will be truly successful upon implementation [260]. It is the moral duty of the researcher to rightly inform the stakeholders, and how research results are portrayed in the media is crucial in forming the public's perception. Although journalists may have a tendency to blur the distinction between what is being experimentally done and what is clinically possible, scientists still have a responsibility to temper such expectations.

The potential of biofabrication may raise concerns of human enhancement practices, as the technology allows control over the architectures of tissues. Yet, body modification has been deeply embedded in our cultures and is actually a product of the evolution of our species. Evolution has caused our species to develop the intellectual capacity to influence our own development, and

through our scientific progress we gain more and more control over this so-called 'neo-evolution' [102]. By treating or preventing disease, we are continuously altering our natural evolution. Although regenerative medicine, to which biofabrication contributes, has the intention to restore tissues and organs to (near-)normal state, [75] the technology could significantly alter and possibly enhance the form, function, and lifespan of an individual. As Fineberg (2011) elegantly states it, "the same engine of science that can produce the changes to prevent disease, will also enable us to adopt superior attributes" [102]. Enhancement by repair paves the way for enhancement of natural features of our body; to not only fix what is broken, but to improve on our exterior, physiological and cognitive features [90]. Human enhancement is not inherently ethically wrong; in fact, we practice it daily by studying to increase our intellectual capacity, by training to become a better athlete, or by wearing glasses to improve our eyesight [163, 216]. Rather, the type of enhancements under debate are those that improve human form or functioning *beyond* what is necessary to restore or sustain health [163]. Aesthetic enhancements, for example, are deemed problematic because they can be intended for the sake of vanity [90]; yet in reconstructive surgery such adaptations are rather made for functional or psychosocial reasons [266]. Highly functionalized tissue-engineered constructs could give rise to performance enhancement intended for greater athletic competitiveness [90], but could just as well have medical applications (*e.g.* a 3D-printed bionic ear where biological tissue is combined with functional electronics for human hearing [212]). A current ethical discussion in regenerative medicine concerns increasing lifespan and longevity by treating conditions due to ageing [353]. Biofabrication-based strategies are currently investigated as interventions for prevention or treatment of degenerative diseases. Taken together, biofabrication technologies have the potential to contribute to changes made to the human body that stir up discussions on human enhancement.

DISCUSSION

Biofabrication is an emerging technology with high potential for increasing the complexity of tissue-engineered products. Developing biofabrication technologies has the potential to improve healthcare by providing highly advanced and personalized biomedical products for research, treatment and prevention. The impacts of emerging technologies on society receive relatively little attention in scientific discussions. However, the inherent relationship between humans and technology [358] requires an integral approach to biomedical innovation. A strive towards coproduction involves a constructive dialogue between science, technology, ethics and society [353]. Involving ethics early in the developmental stages of an intervention allows joint reflections on the objectives, design and impact of the product.

In this review we have highlighted ethical aspects of the translation of regenerative biofabrication technologies from a bench, bedside and societal perspective. This identification of key ethical

topics is meant to serve as an impetus towards a more comprehensive analysis of the ethical implications of biofabrication technologies. Not surprisingly, it appears that there is substantial overlap with the fields of regenerative medicine and tissue engineering, although biofabrication can be set apart by its potential to mass generate highly functionalized and personalized constructs with improved internal and external architectures. In each research stage there are ethical aspects to consider in the development of biofabricated tissue products. In summary, in bench research it is important to consider consent for the use of human materials and the choice of relevant animal models. Upon moving biofabrication technologies towards the clinic, the novelty, complexity and invasiveness of biofabricated products cause substantial uncertainties and risks. It may be preferable to balance risks with potential value instead of individual benefits. In first-in-man trials with biofabricated products, it may be challenging to select appropriate patients and sufficiently inform them on the risks. An important aspect to consider is how the technology affects society. Besides concerns of inappropriate human enhancement and public perception of biofabrication, there are general aspects of introducing any new biomedical technology that are absolutely relevant to consider here too. An expensive innovative technology impacts equity, for example. It is important to develop technologies and products that do not increase social injustice but have the potential to reduce it.

In ethical discussions on societal impacts of a new technology, emphasis is often placed on the hard, quantifiable consequences. However, our lives are constantly shaped by our changing morals and routines, influenced by science and technology. The potential of biofabrication technologies for the creation of tissue-engineered products may, for instance, change perceptions of ownership of human materials, of (the boundaries of) the human body, and of the responsibility towards our bodies [260]. It appears that scientists and physicians do not consider themselves as having the power to alter these impacts [250]. In the constructivist view on technology and society, stakeholders together shape the design of the technology and thus its impact on society [353]. Therefore, it is important that scientists and physicians actively take up their role as an actor, and drive responsible technological innovation in the biomedical field in bench, bedside and society.

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CHAPTER 8

ENGAGING STAKEHOLDERS IN BIOPRINTING RESEARCH: VIEWS AND CONCERNS OF MICROTIA PATIENTS' PARENTS ON BIOPRINTED AURICULAR CARTILAGE

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ABSTRACT

As novel bioprinting technologies are being explored for future auricular cartilage repair, there is also a growing need to involve relevant stakeholders in the research process in order to align research incentives with societal needs and expectations. In line with a Responsible Research and Innovation approach, this study considers patient caretaker views on the development of novel technologies for auricular reconstruction. Questionnaires regarding tissue engineering, bioprinting and stem cell technology were scored on a 5-point Likert-scale by parents of pediatric microtia patients. After a patients/parents information day and subsequent outpatient clinics, 37 questionnaires were returned. The parents expressed an overall positive attitude and receptiveness towards the use of tissue engineering and bioprinting technologies for reconstruction of the auricle. Also, the future possibility of implanting laboratory-made, bioprinted cartilage in their child was received with considerable enthusiasm. However, a contrasting and important finding was parents' reluctance in subjecting their own child to surgical and technological innovations in early stages of the clinical research process, expressing a desire to have more knowledge and preferably proof of success first. This is the first study investigating the attitudes of parents of children with microtia towards biofabrication technologies and underscores the need for their active role in the development of biomedical technologies, from bench to bedside, through a two-way dialogue process.

BACKGROUND

In recent years, bioprinting and stem cell technologies have gained increasing interest as options for regenerative management strategies in medicine [9, 12, 81, 107, 145, 158, 159, 219, 230]. In the field of reconstructive surgery, these methods are progressively being explored for future auricular cartilage repair [159, 266, 340]. Promising results have scientists, physicians and companies express considerable enthusiasm about this perspective [108, 214, 392].

Even though the situation is changing rapidly in the last years, researchers and physicians – with oversight of funding agencies and ethical committees – are still considered the main actors driving (bio)medical research [366]. Further players include companies and policymakers, yet there are also other stakeholders that would require a voice in the development process of new medical and technological products and methods. While scientific citizenship and patient-centered healthcare are deemed increasingly important, the end-user of these innovations, *i.e.* the patient, is often overlooked as a valuable contributor [50, 304, 344, 345]. The research process and its outcomes can greatly benefit from participatory processes when a continuous dialogue between patients and researchers is accomplished [31]. Public engagement in scientific research, and thus also patient involvement in the biomedicine realms, is one of the pillars of the approach adopted by the EU Commission – called Responsible Research and Innovation (RRI) – to align science and society. This framework was designed to overcome misalignment between values, needs and expectations of society to be pursued in Research and Innovation (R&I) and what is actually being researched and developed as innovative products [44, 72, 308, 326, 393].

A remarkable aspect of the RRI approach is that it requires the incorporation of participation of all stakeholders [286]. Patient and public involvement is increasingly present on the political and scientific agenda, and is stimulated by updated regulations of the EU, funding agencies and publishers [366]. The patient is no longer just a 'consumer' of care, but also a contributor. Patients and citizens become equal partners in research and innovation processes. Several examples corroborate this trend. Over the past decades, the doctor-patient relationship has changed from 'paternalistic' towards a more dialogic form involving shared decision-making [47]. Likewise, in biotechnology, there is a shift away from total control in the hands of the producers, towards active involvement of the end-user during the innovation process [308]. Innovation becomes beneficial if it adds value to society according to the needs of its citizens [44]. Especially technologies that have evident social and ethical implications – such as biofabrication – require the acceptance of the public for effective implementation [47]. As such, von Schomberg (2013) argues that "scientific expertise cannot be the sole basis for the development and introduction of new technologies" [308]. Instead, societal actors should become key contributors to research and innovation, with mutual responsiveness and responsibility, and involvement from an early stage onwards [44, 268,

308, 393]. The (early) engagement of users promotes better decision making during the research process, as well as improved applicability of the outcomes [1, 304].

Bioprinting impacts are highly dependent of its effective responsible development [34] and there are many actions that have to be pursued by all the stakeholders involved in the innovation ecosystem in order to enable a responsible development of 3D (bio)printing in the biomedical field [318]. Our current study can be ascribable as one of the first attempts of embedding bioprinting research and clinical applications into an RRI process, involving end-users and stakeholders in the research process of a specific case of bioprinting of auricular cartilage implants. Microtia is a congenital deformity of the external ear that is currently treated with reconstructive surgery using autologous rib cartilage or synthetic polyethylene (Medpor®) implants [384]. As reconstruction is often performed when the patient is still a child, their caretakers become relevant stakeholders. This study explored views and concerns of parents of pediatric microtia patients towards novel technologies – such as tissue engineering, bioprinting and stem cells – for future auricular reconstruction. The objective of this study was to gain insight in the parental views and attitudes towards emerging technologies for future treatment options for microtia, specifically concerning tissue engineering, 3D bioprinting, and related aspects such as the use of (stem) cells, synthetic and natural materials, and their interest of a potential enrollment of their child in the deployment of such techniques. The outcomes of this study help shape future research on biofabrication-based auricular reconstruction in alignment with stakeholder values.

METHODS

Participants were recruited at the annual Microtia/Anotia patients/parents day (October 28, 2017) and subsequent microtia outpatient clinic consultations at the Wilhelmina Children's Hospital in Utrecht, The Netherlands, between October 2017 and June 2018. Participants received oral and/or written explanations about the topics surveyed in the study prior to filling in the questionnaire. Participation was voluntary and anonymous. All documents pertaining to this study were presented to the institutional Medical Research Ethics Committee (MREC) prior to participant recruitment (protocol number 17-744/C). The MREC confirmed that the Medical Research Involving Human Subjects Act (WMO) does not apply to this study, and as such it does not require official approval by the MREC (reference number WAG/mb/17/032289). This study was performed in accordance with current laws and regulations. Informed consent was given by all participants to use their responses for analysis and publication.

A 3-part questionnaire was designed regarding the demographical data of the child and the parents (A), the reactions to the microtia and considerations for surgery (B), and the perspectives on emerging technologies (C). Part A and part B were presented in a mixed format of open questions

and multiple-choice options. Part C consisted of 12 questions in four categories: questions related to technologies, questions related to cells, questions related to materials, and questions related to application. Questions and the associated keywords used throughout this paper are reported in Table 1. Questions were presented in a 5-point Likert-type scale, with 1 meaning a negative attitude towards the topic, 2 being hesitant, 3 being neutral, 4 being receptive, and 5 having a positive attitude towards it. With each question there was room for comments.

Questionnaires were returned by post or during the clinic visit. Returned questionnaires were coded upon reception to maintain anonymity. The results were processed using Microsoft Excel and descriptive analyses are reported here.

Table 1. Categorized questions and associated keywords. Questions were related to technologies, cells, materials and clinical implementation. Keywords describe the topic of the question and are used for short denomination of the question throughout the text and in the results table and figure.

Questions	Keyword
Questions related to technologies	
How do you feel about creating new tissues in the laboratory, in general?	Tissue engineering
How do you feel about the use of laboratory-made cartilage for future treatments for microtia?	Tissue-engineered cartilage
How do you feel about using the new 3D-bioprinting technology for the creation of an ear implant?	3D bioprinting for ear
Questions related to cells	
How do you feel about the use of the patient's own chondrocytes (from the cartilage) for future therapies?	Autologous chondrocytes
How do you feel about the use of the patient's own stem cells for future therapies?	Autologous stem cells
How do you feel about harvesting the patient's own cells from your child?	Harvesting cells from child
Questions related to materials	
How do you feel about the use of synthetic materials for future therapies with tissue regeneration?	Synthetic materials
How do you feel about the use of natural materials for future therapies with tissue regeneration?	Natural materials
How do you feel about the use of cell-seeded MedPor® for ear reconstruction?	Cell-seeded MedPor®
How do you feel about the use of 'decellularized' (donor) tissue?	Decellularized tissue
Questions related to implementation	
How do you feel about the future possibility of implanting laboratory-made cartilage, if this would be in your child?	Implantation of engineered cartilage
How would you feel about the participation of your child in early clinical research?	Participation in early trials

RESULTS

Demographic information

In total, 37 completed questionnaires were returned (Table 2). The respondent was the mother in 57 % of cases, and the father in 27 % of cases. In 5 %, parents completed the same document together. In 11 %, it was unknown who was the respondent. The median age of the mother at time of birth or adoption was 31.5 years (range 20-42 years), and of the father 36 years (range 26-46 years). Education level of mother and father was respectively 27 % and 26 % secondary vocational education, 35 % and 46 % higher professional education, and 38 % and 29 % university level. There were no statistically significant differences between higher and lower educational levels in the mean scores of given answers. The responses involved 28 children, with a median age of four years, ranging from three weeks to 17 years old. The majority of children (71 %) was male. The affected side was right in 54 %, left in 29 %, and bilateral in 18 % of cases. Twenty-one percent of the children had already undergone surgical reconstruction of the auricle, of which 67 % with autologous rib cartilage and 33 % with Medpor®. If the choice was still open, the parents of 18 % of children would choose Medpor®, 14 % would decline surgery, and 68 % had not decided yet.

Technologies

The creation of new tissues in the laboratory was generally anticipated with enthusiasm (Table 3; Figure 1), with 66 % and 26 % of respondents indicating a positive and a receptive attitude, respectively. Five percent was neutral, and 3 % was reluctant. On a scale of 1-5, the median was 5 and the mean score was 4.7 ± 0.6. Similar views were revealed with regards to using laboratory-made cartilage for the treatment of microtia, with 62 % and 24 % of respondents being positive or receptive, 8 % neutral and 5 % reluctant (median 5; mean 4.5 ± 0.7). Respondents were positive (66 %) towards the possibility of using 3D-bioprinting technology for the creation of an ear implant, receptive in 31 % and neutral in 3 % (median 5; mean 4.7 ± 0.5). No one was negative towards the use of these novel technologies for the treatment of microtia. Comments were positive and encouraging, including statements like "beautiful development" and "I'm definitely a proponent of new innovations, especially if it means that surgeries become less intensive." One respondent expressed being "wary of humans 'playing God' and unintended and unimagined consequences, but being encouraged at the same time". Another indicated to be "excited to read about the potential of tissue engineering and bioprinting. Neither rib cartilage or Medpor® are perfect, so we definitely need to find a better alternative. I understand that research is at an early stage, and we still have a long way to go – but I have hope that we can find something fantastic in my son's lifetime."

Table 2. Characteristics of questionnaire respondents and their children with microtia. Information about education levels, affected ear characteristics and treatment preferences was collected.

Respondents		*n = 37*			
Relationship to child	Mother	21	57%		
	Father	10	27%		
	Both	2	5%		
	Unknown	4	11%		
Country of residence	The Netherlands	34	92%		
	United Kingdom	3	8%		
Parents		*n = 37*		median	range
Age at birth / adoption child (years)	Mother			31.5	20 - 42
	Father			36	26 - 46
Education level					
Secondary vocational education	Mother	10	27%		
	Father	9	26%		
Higher professional education	Mother	13	35%		
	Father	16	46%		
University	Mother	14	38%		
	Father	10	29%		
Child		*n = 28*		median	range
Current age (years)				4	0 - 17
Age at diagnosis (years)	<1 year	16	57%		
	1 - 4 years	8	29%		
	5 - 9 years	2	7%		
	≥10 years	1	4%		
	Unknown	1	4%		
Sex	Male	20	71%		
	Female	8	29%		
Affected ear	Left	8	29%		
	Right	15	54%		
	Bilateral	5	18%		
Treatment		*n = 28*			
Child had ear reconstruction	Yes	6	21%		
	No	22	79%		
If yes, type	Rib cartilage	4	67%		
	MedPor®	2	33%		
If no, choice	Rib cartilage	0	0%		
	MedPor®	4	18%		
	No surgery	3	14%		
	Don't know yet	15	68%		

Cells

The use of autologous chondrocytes or stem cells was received with a bit more caution (Table 3; Figure 1). For chondrocytes, 32 % of respondents was positive, 31 % was receptive, 23 % was neutral and 14 % was reluctant (median 4; mean 3.9 ± 0.9). For stem cells, the percentages were 38 %, 26 %, 28 % and 8 % respectively (median 4; mean 4.0 ± 0.9). Respondents expressed concerns about the invasiveness of the harvesting techniques, the pain it would cause and possible scars. For stem cells, concerns were raised about the long-term effects of such cells. When asked about their feelings on harvesting cells from their own child, 30 % of parents indicated to be positive, 19 % to be receptive, 24 % to be neutral, 22 % to be reluctant, and 5 % to be negative (median 3.5; mean 3.5 ± 1.2). A concern that was often expressed was the pain associated with harvesting tissue or cells. Parents indicated that they did not "want to inflict pain in my child if not absolutely necessary." The physical and psychological consequences of the harvest should outweigh the aesthetic advantages of the reconstruction. One respondent stated "I'm less positive towards harvesting cells from my child, but I understand the necessity of using the patient's own material. I would want the least invasive way of reconstruction with the best results." Parents also agreed that their child should have a say in the matter.

Materials

The majority of respondents expressed an openness towards synthetic and natural materials (Table 3; Figure 1). For synthetic materials, 32 % was either positive or receptive, 19 % indicated to be neutral, 15 % to be reluctant and 1 % to be negative (median 4; mean 3.8 ± 1.1). It was regarded as a good alternative to harvesting tissue from the patient (like in auricular reconstruction using rib cartilage), yet concerns were raised about potential rejection by the body. One respondent stated to be "concerned about the durability of man-made materials and how they behave over time".

Natural materials were regarded slightly better, with 46 % being positive, 32 % receptive, 11 % neutral, 9 % reluctant and 1 % negative (median 4.5; mean 4.3 ± 0.9). The possibility of seeding cells on the Medpor® implant was viewed positively in 43 % and 29 % respectively, whereas 19 % was neutral and 8 % was reluctant (median 4; mean 4.1 ± 1.0). One respondent stated that as they were supportive of the Medpor® reconstruction, any development of that towards a more natural outcome would be encouraged. Decellularized tissue was explained, yet many respondents indicated that they would want more information; hence, 33 % indicated to be neutral.

One parent stated to be "not convinced about this as an idea". Nevertheless, 31 % was positive, 23 % receptive, 13 % reluctant, and 0 % negative (median 4; mean 3.7 ± 1.1). One respondent stated that they found it a "scary thought", whereas another said it sounded "feasible".

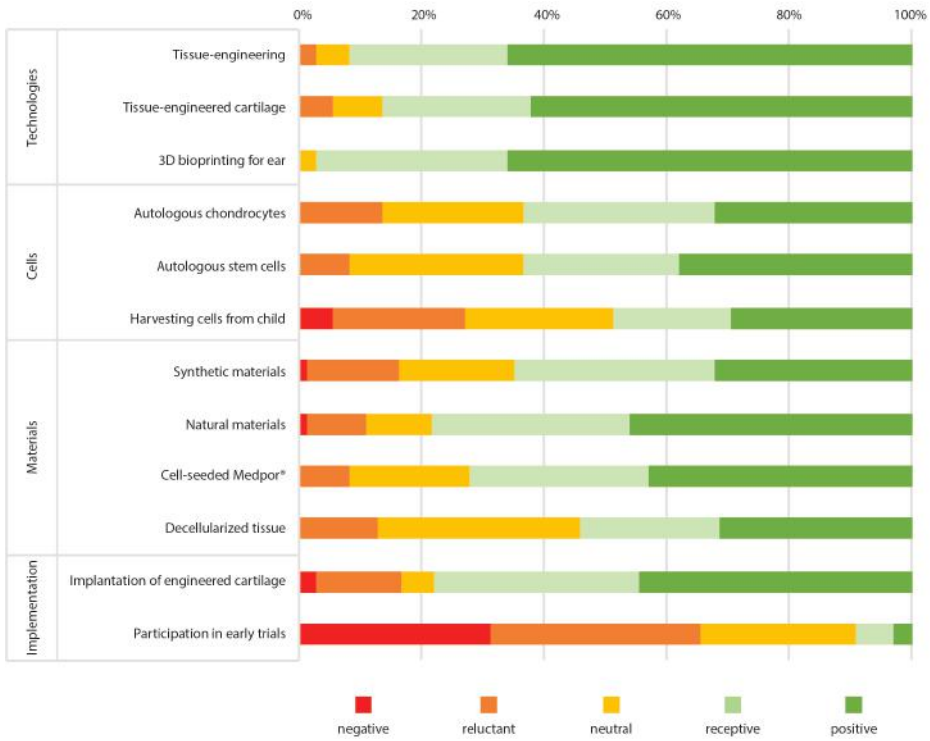

Figure 1. Attitudes of parents of microtia patients towards tissue engineering-based innovations for auricular repair and their translation to the clinic. Negative, reluctant, neutral, receptive and positive responses on questions about technologies, cells, materials and implementation are represented as percentages and expressed as cumulative colored bars.

Implementation

Parents generally expressed positive feelings on the future possibility of implanting laboratory-made cartilage in their child (Table 3; Figure 1). Of the 36 responses, 44 % indicated to be positive and 33 % to be receptive, 6 % to be neutral, 14 % to be reluctant and 3 % to be negative (median 4; mean 4.0 ± 1.1). When asked about the (hypothetical) participation of their child in early-stage clinical trials, only one parent was positive (3 %) and two indicated to be receptive (6 %), stating that "one should be the first anyway. Do as you would have done by others". Of the 32 responses, 34 % indicated to be reluctant and 31 % to be negative about this idea. The majority indicated that they would first want more certainty about the new treatment, especially the risks and long-term effects.

Table 3. Patient caretaker responses to emerging technologies for microtia reconstruction. Responses were reported on a 5-point Likert scale: 1 = negative, 2 = reluctant, 3 = neutral, 4 = receptive, 5 =positive. Both median and mean scores are reported. SD = standard deviation.

				1		2		3		4		5		median	mean	
		responses		negative		reluctant		neutral		receptive		positive		score	score	SD
		n = 37		*n*		*n*		*n*		*n*		*n*				
Technologies	Tissue engineering	37	100%	0	0%	1	3%	2	5%	9.5	26%	24.5	66%	5	4.7	0.6
	Tissue-engineered cartilage	37	100%	0	0%	2	5%	3	8%	9	24%	23	62%	5	4.5	0.7
	3D bioprinting for ear	37	100%	0	0%	0	0%	1	3%	11.5	31%	24.5	66%	5	4.7	0.5
Cells	Autologous chondrocytes	37	100%	0	0%	5	14%	8.5	23%	11.5	31%	12	32%	4	3.9	0.9
	Autologous stem cells	37	100%	0	0%	3	8%	10.5	28%	9.5	26%	14	38%	4	4.0	0.9
	Harvesting cells from child	37	100%	2	5%	8	22%	9	24%	7	19%	11	30%	3.5	3.5	1.2
Materials	Synthetic materials	37	100%	0.5	1%	5.5	15%	7	19%	12	32%	12	32%	4	3.8	1.1
	Natural materials	37	100%	0.5	1%	3.5	9%	4	11%	12	32%	17	46%	4.5	4.3	0.9
	Cell-seeded MedPor®	36	97%	0	0%	3	8%	7	19%	10.5	29%	15.5	43%	4	4.1	1.0
	Decellularized tissue	35	95%	0	0%	4.5	13%	11.5	33%	8	23%	11	31%	4	3.7	1.1
Implementation	Implantation	36	97%	1	3%	5	14%	2	6%	12	33%	16	44%	4	4.0	1.1
	Participation in early trials	32	86%	10	31%	11	34%	8	25%	2	6%	1	3%	2	2.2	1.1

DISCUSSION

As bioprinting and stem cell technologies demonstrate increasing potency for application in reconstructive surgery, it also becomes more important to involve relevant stakeholders since the very outset of the development of novel strategies for treatment. In line with a Responsible Research and Innovation approach, both the research process as well as its outcomes should be aligned with the values, needs and expectations of our society [268, 308, 326, 393]. An important pillar of RRI is the (timely) participation of all relevant stakeholders [286] and the interactive quality of the entire process [308]. As such, without the (active) involvement of the stakeholder that is the end-user, *i.e.* the patient, there may be a mismatch between research incentives and medical need [72, 140]. If the patient is not receptive to the innovation, it's a loss of valuable resources that could otherwise have been avoided. The goal of RRI is to avoid such a mismatch between research incentives and medical and societal needs, and to apply valuable resources in the most effective manner [140].

Despite increasing efforts to involve relevant stakeholders in the research process, it appears that there is an important incongruity between research priorities of investigators and funders on the one hand, and clinicians and patients on the other hand [72]. In fact, even if parents of children with microtia are crucial stakeholders, their views and opinions are still often overlooked. This study aimed to initiate the involvement of the end-user in the research process of bioprinting cartilage for auricular repair and to start exploring patients' and caretakers' interest in and concerns on related clinical trials.

The results from our study indicate an overall positive attitude and receptiveness of parents towards the use of novel regenerative medicine technologies such as tissue engineering, bioprinting and stem cell technology for reconstruction of the auricle. The future possibility of implanting laboratory-made, bioprinted cartilage in their child was received with considerable enthusiasm. However, a contrasting and important finding was parents' reluctance in subjecting their own child to surgical and technological innovations and enrolling them in early stages of the clinical research process, expressing a desire to have more knowledge and preferably proof of success first.

In our case of microtia reconstruction, which is most commonly performed in children, classic means of informed consent of both child and responsible caretaker are not sufficient. Novel forms of participation and engagement need to be designed and implemented to achieve an effective responsible development of bioprinting towards application in the clinic. At the same time, it becomes clear that future users of biofabrication applications require extensive information about the technology, the benefits and the risks before choosing to use the innovation. Especially in

the case of microtia reconstruction, specific education and informed consent of both child and responsible caretaker is crucial. Currently, as emerged from the results of our study, parents of children with microtia still portray hesitant attitudes towards their child's potential participation in a clinical trial; yet our preliminary study doesn't offer clear evidence if this attitude is related or not to the specific technology involved. More specific insight in these attitudes and concerns about the process could help shape the design of future clinical trials with biofabricated cartilage for auricular reconstruction, fully addressing a participatory approach toward effective patient engagement and empowerment.

With the rise of patient-centered medicine as well as online access to medical information, patients demand more autonomy and responsibility over their own health. Patients increasingly participate in decision-making regarding their health management. However, this should also require their participation in the research that leads to health management options [304, 345]. There is a general strong belief in science and its benefits, yet the public does not passively accept developments in science, technology and medicine [47, 221]. Concerns are raised by the public about impacts and risks of novel (bio)technologies, and their assessments often include the degree of naturality, morality and ethicality. There is increasing evidence that members of the public are able of thinking critically about scientific discoveries. In addition, they seem to assess pragmatically and judge new technologies in context [47]. As such, the inputs of the lay public and patients can be highly valuable as they can lead to new perspectives for researchers and co-innovators [1, 304]. This underscores the need for their active role in the development of biomedical technologies, through a two-way dialogue process.

CONCLUSION

Parents of patients with microtia expressed an overall positive attitude and receptiveness towards the use of tissue engineering and bioprinting technologies for reconstruction of the auricle. Nevertheless, they indicated reservations to the idea of subjecting their own child to surgical and technological innovations in early stages of the clinical research process, expressing a desire for more knowledge and proof of success first. This study takes a first step towards involving patient (caretaker) stakeholders, taking their views seriously into account and stimulating a two-way dialogue about the innovation of auricular reconstruction using novel technologies and potential clinical applications and trials.

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DISCUSSION

CHAPTER 9

SUMMARIZING DISCUSSION AND FUTURE PERSPECTIVES

SUMMARIZING DISCUSSION

Current strategies for the reconstruction of the human auricle can benefit from a regenerative approach that involves the use of autologous cells in combination with supporting biomaterials for the generation of auricular cartilage. The aim of this thesis was to explore the challenges in bioengineering an auricular cartilage implant and to provide potential solutions for overcoming these challenges. **Chapter 2** reviewed the development of auricular cartilage engineering over the past decades, from diced cartilage grafts in molds via cell-seeded mesh scaffolds to hydrogel-based constructs. The main challenges deduced from this historical perspective include the generation of a functional biochemical matrix, the fabrication of a personalized anatomical shape, and the long-term maintenance of shape, size and tissue quality of the engineered auricle.

The strategy adopted in this thesis to bioengineer a human auricular cartilage construct includes clinically scalable regenerative cartilage progenitor cells, bioprinting for increased spatial control, fiber reinforcement for shape preservation, and smart scaffold design for improved nutrient diffusion and enhanced anatomical details. **Part I** focused on finding an appropriate cell source for engineering the human auricle. The auricular cartilage progenitor cells identified in this thesis can be extensively multiplied to acquire sufficient cells for constructs of clinically relevant sizes and maintain a high cartilage regenerative potential after expansion, thereby providing an opportunity to progress auricular cartilage tissue engineering. In **Part II**, the need for improved mechanical integrity of engineered auricular constructs was underscored. Biofabrication enables the generation of customized multi-material architectures with high control over the internal spatial organization, thereby allowing the incorporation of tunable reinforcing networks. Addressing mechanical reinforcement, optimized nutrient diffusion and construct scalability, a hybrid fiber-reinforced auricular construct was bioprinted. The auricular structure closely resembled the digital 3D model and maintained that shape during chondrogenic pre-culture, during which abundant cartilage-specific matrix was produced by the embedded progenitor cells. This proof-of-concept shows that a combination of various strategies may be able to advance auricular bioengineering towards a clinically viable option. The emergence of regenerative biofabrication technologies can have significant impacts on future therapeutic and preventative interventions. Therefore, **Part III** placed biofabrication research into a societal context in order to contribute to a responsible research process. Challenges in designing appropriate clinical trials with disruptive technologies such as biofabrication are discussed, and attention is drawn to public expectations, societal impacts and stakeholder involvement. Embracing ethics as a valuable guide in the research process will enable the biomedical research community to align research incentives with societal needs and values.

PART I – CELLS: FINDING AN APPROPRIATE CELL SOURCE FOR ENGINEERING THE HUMAN AURICLE

The engineering of cartilage-like tissue has been demonstrated using chondrocytes sourced from auricular, articular, nasoseptal and costal cartilage [27, 49, 155, 169, 185, 224, 240]. Nevertheless, it remains a significant challenge to recruit a sufficient number of cells for the generation of large tissue constructs. Estimates for a full-size human auricle range between 100 million and 250 million cells [25, 66]. Chondrocytes exhibit a naturally low proliferative potential [288] and dedifferentiate after extended cultivation [303], resulting in a more fibrocartilage-like matrix with inferior biochemical and biomechanical properties compared to native cartilage tissue [63, 80, 234]. Redifferentiation can to some extent be achieved under specific culture conditions, *e.g.* in three-dimensional (3D) environments [19] and in the presence of appropriate growth factors [156, 355], yet the revenue is limited [355]. Another commonly used cell type for cartilage engineering are mesenchymal stromal cells (MSC), derived from bone marrow, adipose tissue or other sources [2, 313, 359]. MSCs have an extensive capacity for self-renewal without losing multi-lineage differentiation potential [117, 290] and they have successfully been applied for the generation of cartilage-like tissue [60, 292, 313, 359]. Nevertheless, the tendency of these cells to undergo hypertrophic growth that can result in tissue calcification and increased implant rigidity is an undesirable risk [120, 229].

Tissue-derived stem/progenitor cells exhibit stem-cell like qualities, including self-renewal and multipotent differentiation capacity, yet are embedded in the target tissue niche and are primed to differentiate towards that tissue [157]. **Chapter 3** identified a resident progenitor cell population in the equine auricular cartilage and compared their cartilage regenerative potential with auricular chondrocytes and MSCs. Encapsulated in gelatin methacryloyl (gelMA) hydrogels, auricular cartilage progenitor cells (AuCPC) demonstrated the ability to synthesize abundant cartilage-specific components, such as proteoglycans and collagens, after *in vitro* chondrogenic stimulation. These results provided a new avenue for the generation of auricular cartilage constructs. In **Chapter 4**, the presence of these AuCPCs was subsequently confirmed in human adult auricular cartilage, healthy pediatric auricular cartilage and rudimentary cartilage remnants from pediatric patients with microtia. Proliferation and differentiation assays determined that AuCPCs can be extensively expanded without losing multi-lineage differentiation potential. *In vitro* experiments demonstrated that human AuCPCs produced cartilage-specific matrix in hydrogel constructs.

Cell selection is a crucial aspect of successfully generating cartilage tissue constructs of clinically relevant sizes. Autologous AuCPCs can easily be obtained through a non-deforming biopsy of the normal ear or from the rudimentary microtia cartilage, thereby limiting the invasiveness of harvesting cells. The isolated cells have the ability to supply the required cell numbers for tissue

engineering of an auricular cartilage implant and can successfully be applied for the generation of auricular cartilage. The potency of AuCPCs can potentially be boosted by optimizing culturing conditions in order to stimulate matrix deposition specific to the auricular cartilage phenotype. Growth factors of interest that promote elastin production include CCN2/CTGF [111], insulin [300] and IGF-1 [300], and possibly a transient exposure to TGF-ß1 [249]. In addition, future studies could investigate a long-term comparison between available cell sources and the behavior of AuCPCs in an *in vivo* setting. The current results indicate that using AuCPCs for cartilage regeneration can overcome donor site morbidity, cell dedifferentiation and matrix calcification. Hence, AuCPCs can provide an important solution to long-existing challenges in auricular cartilage tissue engineering and may aid in translating the technology towards clinical application.

PART II – FABRICATION: IN SEARCH OF A DURABLE AURICULAR IMPLANT SHAPE

A durable engineered auricular implant requires inherent strength and stability in order to be successful in the long term. This means that cartilage matrix deposition should be abundant and appropriately organized to properly mimic the native tissue's microscopic anatomy and biomechanical properties. With control over the spatial organization of cells and matrix components, biofabrication can create a blueprint of a tissue or an organ. In the subsequent maturation phase, however, the exact development of that tissue or organ is dependent on the performance of cells in relation to their environment [191]. An *in vitro* pre-culture period allows for some degree of cellular guidance and stimulation before the engineered construct is implanted in the complex *in vivo* domain. After implantation, the construct is under the influence of systemic and local biochemical processes. In the case of the auricle, which is located at the outside of the cranium, both internal and external forces are at play as well. Engineered auricular constructs will be implanted under the cranial skin, which is a highly viscoelastic tissue that has high mechanical restraining capabilities [91, 149]. With increasing strain, the skin offers more resistance and presses on the underlying material. These contraction forces may lead to problems with the engineered auricular implant [17, 389]. Previous studies that implanted engineered auricular cartilage constructs under the dorsal skin of mice encountered deformation and collapse of the structures [49, 295, 315]. The cranial skin in humans is thicker and stiffer than murine skin and microtia patients have less skin available as the auricle has not developed fully. As such, the skin envelope that is available for auricular implants may be especially tight.

Chapter 5 determined the skin surface area of microtia auricles and the normal contralateral ear in patients with microtia. While presenting a novel method to calculate skin coverage using micro-computed tomography (µCT) scanning and 3D mesh processing, this study found a significant skin deficiency of >50 % on the microtia side compared to the normal auricle. Skin coverage is crucial

for successful reconstruction of the auricle, and these results show that there is very limited skin available for coverage of an auricular implant. The provision of sufficient skin can improve healing after auricular reconstruction and reduce excessive forces on the developing cartilage [17, 389]. Extra skin can be made available through tissue expansion, flap transposition or skin grafts [178, 199, 381]. Nevertheless, remaining contractive skin forces as well as daily external influences, such as sleeping and wearing a helmet, will impact the development and durability of the engineered implant. Therefore, improvement of mechanical stability of the engineered auricle is an important point of attention in auricular cartilage tissue engineering.

The need for mechanical support of developing auricular constructs has been recognized early on, and stents and molds ensured initial support of the auricular structure [49, 168, 246, 373]. Nevertheless, external fixation is not a durable option as its inevitable removal is likely to result in shrinkage and deformation of the still immature neocartilage [49]. The implantation of engineered auricular constructs with internal supporting structures has yielded better outcomes in terms of maintaining dimensions and contours [54, 136, 167, 201, 376, 389, 391]. Through biofabrication technology, reinforcing scaffolds can be generated with control over architecture, porosity and spatial distribution. As such, complex structures with tailorable mechanical properties can be fabricated. The reinforcement of hydrogel-based constructs with bioprinted polymer fiber networks has been demonstrated to significantly increase the biomechanical characteristics of engineered cartilage [16, 28, 363].

Polymer scaffold requirements for regenerative medicine purposes have been previously postulated to include a highly porous interconnected network to allow cell growth and the transport of nutrients and waste products, with mechanical properties matching the native tissue, that is made of a material with appropriate surface chemistry to support cellular function, and that is biocompatible and controllably resorbable [150]. The biodegradable polymer poly-ε-caprolactone (PCL) has been described as a highly suitable platform for tissue engineering applications [370]. It is a synthetic thermoplastic polymer that has a longstanding history in medical use in FDA-approved sutures and drug delivery devices [370, 380]. The material is bioresorbable through hydrolysis and the degradation products are excreted without accumulation in the body [330, 370, 380]. Biodegradability *in vivo* has been observed as a decrease in molecular weight without loss of shape over the course of two years [330]. PCL has previously been applied for the fabrication and reinforcement of various complex structures, including the human auricle [165, 170, 190, 278, 361, 364, 388, 391].

In **Chapter 6**, hybrid auricular constructs were fabricated, composed of gelMA hydrogel laden with human AuCPCs and reinforced with PCL, using biofabrication technologies. This approach addressed several challenges in auricular cartilage engineering: cell source, mechanical integrity,

auricular shape creation and preservation, and nutrient limitation. AuCPCs have previously demonstrated adequate cartilage-like matrix production in gelMA hydrogels but had not yet been applied for the engineering of auricular structures. This study was the first to utilize these cells for biofabrication purposes. The results show that human AuCPCs were not affected by the bioprinting process in terms of viability, metabolic activity and cartilage-like matrix production. Taking into account the outcomes of **Chapters 3**, **4** and **6**, this resident progenitor population in auricular cartilage is a promising candidate for the bioengineering of the human auricle.

Fused Deposition Modelling (FDM) of PCL fiber networks was applied for reinforcement of cell-laden hydrogel structures to improve mechanical properties. The incorporation of stabilizing PCL fibers did not negatively affect the ability of AuCPCs to produce glycosaminoglycans and aligned the compressive modulus of fabricated constructs with that of native auricular cartilage. Enhancing mechanical strength is essential for the durable survival of the engineered auricle, yet it is also imperative that reinforcing scaffolds should not become too rigid, as this may cause discomfort and pain, and potentially also soft tissue inflammation, skin necrosis and implant exposure or extrusion even after light traumas [73, 110, 159]. Therefore, it is important to create reinforcement that provides both compressive strength as well as flexibility. Future research on auricular cartilage engineering would benefit from the inclusion of 3-point bending tests to asses bending behavior of the reinforcing scaffolds. Such quantitative data would help in designing reinforcing structures with more refined mechanical attributes. An interesting option would be to incorporate organized microfibrous 3D PCL meshes fabricated through the Melt Electrowriting (MEW) technique into hybrid auricular constructs. These fibers have been demonstrated to markedly increase the compressive and shear properties of hydrogel-thermoplastic constructs [16, 52, 78, 363] and may allow improved flexibility of engineered auricular constructs without compromising on other key features such as stiffness.

A hybrid bioprinting technique enabled the precise fabrication of 3D auricular structures in **Chapter 6**, with excellent scaffold porosity as well as shape fidelity compared to the digital model. Accuracy of the auricular shape in relation to the original design was very high directly after printing, and size and shape were maintained during a 30-day dynamic *in vitro* culture. The PCL network appears to provide adequate construct stabilization during a pre-culture period prior to implantation. As the compressive properties of the scaffolds used in this study are in the same range as native auricular cartilage, it is likely that it may also be able to withstand forces that can be encountered *in vivo*. Nevertheless, scaffold optimization, more elaborate mechanical evaluation and *in vivo* studies are required before translation to clinical practice.

The use of biofabrication technologies is not only beneficial for precise control over the internal organization of a hybrid construct, it also allows the creation of complex, patient-specific and

customized shapes. The ear is as unique as a fingerprint [147] and successful auricular reconstruction requires the fabrication of an aesthetically pleasing, patient-specific shape. Accurately mimicking the unique and complex shape of the human auricle is a major challenge in auricular reconstruction as well as tissue engineering [17, 25, 266]. Computer-aided design and manufacturing (CAD/CAM) techniques can precisely determine the auricular shape of the normal contralateral ear of a patient and transform the 3D image data into a manufacturing output file for bioprinting [14, 33, 122, 241, 295]. Nevertheless, the resulting full-thickness auricular implants generally have a less pronounced appearance after implantation under the skin. Current surgical strategy overcomes this by using an open framework that omits areas in the scapha, fossa triangularis and concha. By emphasizing the natural eminences and depressions of the auricle, this strategy takes into account the thickness of the skin and facial flaps that are used to cover the auricular implant. The design that was proposed in **Chapter 2** and that was used for the fabrication of an auricular structure in **Chapter 6** used this open framework approach in order to preserve the engineered auricle's anatomical details after implantation, resulting in a satisfactory aesthetic appearance under the skin.

The open framework design has the additional benefit that it aids in improving adequate nutrient supply. The auricular cartilage is a naturally avascular tissue and the nutrient/waste exchange occurs through the process of diffusion. Cellular metabolism mainly requires oxygen for mitochondrial respiration and glucose for glycolysis for the production of ATP. The majority of the chondrocyte's energy requirements is provided by glycolysis and resident chondrocytes in mature cartilage tissue are able to exist in an environment with a low oxygen tension [6]. However, actively differentiating cells in immature engineered tissue constructs exhibit a much higher nutrient requirement [208]. As engineered cartilage is dependent on the diffusion of oxygen and nutrients from the surface into the tissue, cells at the periphery will have an evident advantage over the more distant cells in the center of the engineered construct [231]. The process of diffusion moves a substrate from an area with a higher concentration to an area with a lower concentration. Cells competing for nutrients at the periphery of engineered constructs will therefore have access to a greater supply than cells in the center. Indeed, **Chapters 3** and **4** observed a more pronounced deposition of glycosaminoglycans at the periphery of cylindrical hydrogel samples, with less to no matrix production in the center. In addition, deformation and collapse have been reported in studies with full-thickness engineered auricular structures [49, 295, 315], which has been attributed to central cell death. We performed additional experiments in which it was verified that human AuCPCs are dependent on both glucose and oxygen (**Annex I**) for cellular health and tissue synthesis. Inhibition of glycolysis and mitochondrial respiration both resulted in a significant impairment of GAG synthesis. Cellular consumption of nutrients has been deemed primarily responsible for the creation of spatial gradients in nutrient concentrations throughout hydrogels [43]. As fluorescent-labelled glucose was able to penetrate the hydrogel samples fully in this experiment, it is unlikely that glucose deprivation is the main limiting factor behind central cell death in gelMA hydrogel

constructs. In *in vitro* culture conditions, water-soluble glucose is abundantly present in the culture media and its diffusion appears not to be limited by cellular consumption [101]. Oxygen has a concentration of 18 % in culture media under standard culture conditions [248]. However, because of the low solubility of oxygen, its consumption by peripheral cells results in concentration gradients that become more pronounced with increasing cell densities, even resulting in hypoxia or anoxia in the center of engineered constructs [208]. When the oxygen tension in the center is too low to support viable cells, these cells will undergo apoptosis with central necrosis in the construct as a result [231]. A maximum thickness of <2 mm has been postulated to be the limit for diffusion in engineered constructs [131]. As such, diffusion by itself will be unable to accommodate the metabolic demand of the cells in the more central zones of larger engineered constructs [231]. The modular design proposed in **Chapter 2** divides the larger auricular structure into smaller parts and by applying the open framework approach the maximum thickness of the framework is 2 mm, thereby reducing the diffusion distances for nutrients. In addition, the construct's surface area for diffusion is maximized in comparison to full-thickness auricular constructs, allowing more nutrients to enter the construct. This way, an increased provision of nutrients at the surface of the construct as well as shorter diffusion distances could improve cellular performance in both peripheral and central zones of larger engineered constructs. After an initial maturation phase, modules can be attached and integrated through sutures, adhesives [23] and/or surface degradation [258] to form the complete structure that can be implanted in patients. In **Chapter 6**, abundant cartilage-like matrix deposition was observed throughout the auricular constructs, indicating improved nutrient delivery to central areas. Nevertheless, an inhomogeneous distribution of cartilage components was still observed, and additional strategies for overcoming nutrient limitation and stimulation cellular performance may be required.

One such strategy, although non-reflective of native auricular microanatomy, would be to create perfusion channels to allow non-obstructed flow of nutrients into the construct [198]. Microchannel networks have been successfully created using biofabrication technologies [10, 22, 114] and their effectiveness in cartilage regeneration has been repeatedly demonstrated [43, 64, 174]. Kang *et al.* (2016) reported improved cartilaginous matrix formation throughout auricular constructs due to the incorporation of microchannels [170]. The incorporation of microchannels thus enhances nutrient delivery by shortening diffusion distances and allowing dynamic perfusion.

Another interesting strategy would be to provide a reservoir of nutrients within the engineered constructs to alleviate metabolic stresses during periods of high nutrient requirement. Oxygenating species, such as calcium peroxide [261] or sodium percarbonate [139], can be incorporated into biomaterials and provide oxygen through a gradual decomposition reaction. Alternatively, oxygen-carrying myoglobin complexes conjugated onto the plasma membrane release oxygen responsively to hypoxic conditions and can thereby alleviate severe hypoxia

in central areas of large tissue-engineered constructs [7]. Such innovative approaches can tremendously improve cell survival and tissue development in large engineered constructs like the auricle. We initiated experiments using the cell-supporting hydrogel as a carrier for extra nutrients (**Annex II**). Throughout this thesis, the hydrogel gelatin methacryloyl (gelMA) was used as a cell carrier and bioink. GelMA is biocompatible, tailorable and biodegradable and has inherent bioactivity facilitating cellular function [177]. This hydrogel has been shown to be a conducive environment for chondrogenesis [193, 194, 227, 312] and has demonstrated support of differentiating cartilage progenitor cells [193, 265]. Moreover, gelMA can be modified and functionalized to comply with processing requirements or to stimulate tissue development [177]. In our preliminary experiments, we aimed to incorporate myoglobin into the hydrogel in order to provide an oxygen reservoir. Myoglobin is an important oxygen carrier in the body that normally serves as an oxygen storage protein. It is present in muscle and has a higher affinity for oxygen than hemoglobin circulating in the blood, only releasing its bound oxygen upon severe hypoxic circumstances. GelMA functionalized with tyramine (gelMA-Tyr) allows for the covalent interaction between tyramine groups in the gel and tyrosine residues in proteins such as myoglobin. The results from the experiments in **Annex II** show that myoglobin diffuses out of gelMA hydrogel samples, whereas the protein can successfully be immobilized within gelMA-Tyr hydrogels. An important note to these observations is that the myoglobin used in these preliminary experiments was deoxygenated. Myoglobin can be oxygenated in the presence of a reducing agent, such as sodium dithionate. Nevertheless, these preliminary results show promise for the incorporation of oxygen in hydrogels. Oxygen remains bound to myoglobin under mild hypoxia and is only released when the oxygen tension is critically low. As such, oxygenated myoglobin molecules immobilized throughout the hydrogel can serve as reservoirs for severe hypoxic circumstances and thereby prolong cell viability, improve cell function and promote cartilage matrix deposition in central areas of large tissue-engineered constructs, such as the human auricle.

Taken together, auricular structures can reliably be fabricated using biofabrication technologies. Engineered auricular cartilage constructs of clinically-relevant size and quality could benefit from optimized culture conditions, fiber reinforcement, the incorporation of microchannels, the provision of internal nutrient reservoirs, and a modular fabrication and maturation approach.

PART III – SOCIETY: BIOFABRICATION RESEARCH IN AN ETHICAL CONTEXT

Before biofabrication-based auricular tissue engineering can become a viable option as a therapeutic intervention, there are several issues to be addressed. At the bench level, enhanced tissue formation, improvements in construct architecture on the micro-scale, and optimization of the process of personalized construct fabrication would be required. In addition, clinical use

of tissue-engineered implants demands defined circumstances in order to guarantee patient safety. It necessitates the use of serum-free culture media and the avoidance of animal-derived components [247, 282], as well as the application of biocompatible, biodegradable and endotoxin-free bioinks [166]. *In vitro* optimization with subsequent long-term *in vivo* studies in relevant animal models to asses efficacy and safety are important steps towards realization of clinical translation of biofabricated implants.

It is imperative to anticipate this translation of biofabricated 3D products into patients and society from an ethical perspective. Although ethics is sometimes regarded as a brake on scientific progress, ethics in parallel with research can effectively anticipate societal impacts of emerging technologies and stimulate responsible innovation from an early stage onwards. **Chapter 7** reviews important points of existing ethical discussions in bench and bedside research, and calls attention to emerging issues specific to 3D biofabrication and its translation to society.

In bench research, it is important to consider the appropriate acquisition of human materials, their storage in biobanks and proper consent for use. Upon moving biofabrication technologies to the clinical research phase, the novelty, complexity and invasiveness of biofabricated products require specific refinement of the standard ethical, legal and regulatory framework of clinical trials. The dynamic interaction between the body and the engineered implant is regarded as a major challenge in determining possible outcomes [348]. Due to the lack of prior comparators in tissue engineering to base anticipated risks and benefits on, as well as the variability and complexity of the product, the uncertainties and (un)known unknowns are substantial for biofabricated products [251]. Does the novelty and the potential of the biofabrication field grant acceptance of higher risks and more uncertainties? While risks to participants must be proportional to the anticipated benefits, it may be preferable in young innovative fields to balance risks with potential value instead of individual benefits [134]. In first-in-man trials, it can be challenging to select appropriate patients for participation in the study and to sufficiently inform them of these risks. High expectations of the field may cause people to regard biofabrication as a magical solution for difficult medical problems, and potential study participants may be prone to therapeutic misconception [251].

Emerging technologies and scientific progress generally spark excitement. Inflated expectations, however, can lead to public disillusionment as a field fails to deliver on the anticipated timeline, and unrealistic promises can thus severely damage a field's reputation. Public trust can be earned by presenting concrete steps on the way to the proverbial flag on the hill – the ultimate goal of application of a biofabricated product – and by keeping stakeholders informed [259]. Scientific citizenship is the increasingly important ideal that the public is well informed and able to make decisions regarding scientific research [124]. The involvement of members of the public or patient stakeholders early on can promote better decision making during the research process and

can improve the applicability of the outcomes [1, 304]. This requires an understanding of the research process, including scientific philosophy, funding, experimental phases, timeline, hype, failure, as well as a certain degree of knowledge of the content of the research. Stakeholders with sufficient awareness of the potential impact of a technology on their lives, on a realistic timeline, can provide the researcher with valuable input on the values and needs of society, the degree of public acceptance, the aspects of the technology people are resistant to, and how a technology can be refined so it will be truly successful upon implementation [260]. However, the voice of public and patient stakeholders is often absent in research design, planning and conduct. A potential reason for this may be a lack of trust in the capabilities of the lay public. But whereas a decade ago civil scientific literacy was deemed too low for qualitative participation, the efforts of scientists to enhance public scientific literacy through informal education (*e.g.* museum expositions, popular science books, television shows, newspaper articles, documentaries) are having a positive effect. High percentages of the public are reported to be interested in science and new medical discoveries, and civil scientific literacy is increasing [221]. In addition, the doctor-patient relationship has also changed in the past decades, from 'paternalistic' towards a more dialogic form involving shared decision-making [47]. Patients and the public do not passively accept assigned therapeutic options and developments in science, technology and medicine anymore [47, 221]. There is a general strong belief in science and its benefits, yet concerns are raised about impacts and risks of novel (bio)technologies, and their assessments often include the degree of naturality, morality and ethicality. There is increasing evidence that members of the public are able of thinking critically about scientific discoveries, and they seem to assess pragmatically and judge new technologies in a societal context [47]. In times where technologies are evolving rapidly and societal norms and values are under pressure, it is especially important to recognize patient and public stakeholders as valuable contributors to the scientific process. In fact, researchers, clinicians and institutions now have an ethical responsibility – commissioned by the European Union's call for Responsible Research & Innovation (RRI) – to include members of the public in the research dialogue. The RRI framework is designed to overcome misalignment between values, needs and expectations of society and what is actually being researched and developed as innovative products [44, 72, 308, 326]. Although it appears that clinicians and researchers do not regard themselves as powerful actors in this realm [250], it is important that this role is being recognized and actively engaged in [264].

For this reason, **Chapter 8** evaluates the views of patient caretakers on biofabrication-based tissue engineering technologies. Questionnaires regarding tissue engineering, bioprinting and stem cell technology were scored on a 5-point Likert-scale by parents of pediatric microtia patients. The parents expressed an overall positive attitude and receptiveness towards the use of tissue engineering and bioprinting technologies for reconstruction of the auricle. Also, the future possibility of implanting laboratory-made, bioprinted cartilage in their child was received with

considerable enthusiasm. However, a contrasting and important finding was parents' reluctance in subjecting their own child to surgical and technological innovations in early stages of the clinical research process, expressing a desire to have more knowledge and preferably proof of success first. The results underscore the need for their active role in the development of biomedical technologies, from bench to bedside, through a two-way dialogue process.

The involvement of patient caretakers also revealed concerns about the potential unintended or unimagined consequences of applying regenerative biofabrication technologies in humans. One respondent found it "a scary thought", and another expressed being "wary of humans playing God". Adaptations to human form and functioning beyond what is necessary to restore or sustain health are often a cause for debate [163], but human enhancement is not inherently wrong; in fact, it is part of our history and nature [42]. Human beings wear glasses to improve eyesight, study to improve cognitive abilities, train to become a better athlete, use wearables to gain insight in performance, and create tools and medicines that enhance daily functioning. Concerns may refer to a perceived interference with the evolutionary process, yet the fact that our species has obtained the ability to use our intelligence to deliberately adapt our own biology is a product of that same evolutionary process [102, 163]. Our species is gaining increasing control over our own biological substratum and can change it deliberately, in accordance with values and based on scientific knowledge [42].

Contemporary medicine is already increasing the duration and quality of life through medicines, therapies and surgical interventions. Although regenerative medicine has the intention of restoring tissues and organs to a (near-)normal state [75], it also provides the possibility to exceed the boundary of what is currently considered normal, natural and desired. Biofabrication-based tissue engineering strategies are being investigated as interventions for the prevention or treatment of degenerative diseases (*e.g.* osteoarthritis) or congenital malformations (*e.g.* microtia). The technology allows increasing control over the spatial organization and content of a tissue construct, and therefore has the potential to significantly alter its form and function. For example, it can incorporate components that add functionality to a tissue or organ. An illustrative case is the 3D-printed bionic ear that combined biological tissue with functional electronics that could restore hearing in microtia patients with conductive hearing loss. The bionic ear was able to perceive auditory signals in the normal human range (20 hertz to 20 kilohertz), as well as beyond (frequencies in the megahertz to gigahertz range) [212]. Although intended for functional repair of existing faculties, such innovations can also give rise to performance or cognitive enhancement by providing superhuman abilities.

As discussed in **Chapter 7**, enhancement by repair paves the way for enhancement of the natural characteristics of the body – to not only fix what is broken, but to improve our exterior,

physiological and cognitive features [90]. Besides biofabrication, technologies like gene editing, nanotechnology, artificial intelligence and brain-computer interfaces are innovations that can contribute to the creation of 'superhumans'. To many, it may sound like science fiction; yet the exponential development of science and technology is making the transhumanist perspective more and more a reality. Bioengineering is surpassing natural selection and through alterations and additions may be able to overcome current human limitations. In fact, it may turn *Homo sapiens* into something different – *Homo deus*, a superior version with upgraded physical and mental abilities [137].

But is that wrong, in and of itself? Biomedical enhancements are intrinsically not a pursuit of perfection or mastery, but of well-intended improvements in the quality of our lives. Enhancement is part of human evolution [42]. Yet a valid concern, as posed by a respondent in **Chapter 8**, is whether the involved technologies can give rise to unintended bad consequences. Potential biological, psychological, social and moral harms need to be taken seriously. When properly understood, these concerns can provide valuable guidance in the responsible exploration of biomedical enhancements [42]. This underscores the importance of the RRI framework and morally demands the biomedical research community to include the ethical perspective in research planning and conduct.

CONCLUSION

This thesis addressed the main challenges in bioengineering an auricular cartilage implant for ear reconstruction, in the domains of cells, fabrication and society. These challenges included the identification of an appropriate autologous cell source for the generation of high quality neocartilage of clinically-relevant sizes, the creation and maintenance of a patient-specific and durable auricular shape, and the ethical factors associated with conducting research on innovative and potentially disruptive technologies. Novel cartilage progenitor cells were identified in the auricular cartilage, characterized as potent regenerative cells, and could successfully be incorporated in bioprinting approaches for auricular tissue engineering. Reinforcing structures for improved mechanical stability were fabricated in customized auricular shapes for improved aesthetic appearance and increased nutrient diffusion. The combination of smart scaffold design, potent regenerative progenitor cells, intricate mechanical reinforcement and innovative nutrient delivery strategies can pave the way towards clinical translation of bioengineered auricular cartilage implants. It is imperative that future research is conducted in parallel with in-depth ethical analysis in order to guarantee responsible innovation that is aligned with the values and needs of society.

APPENDICES

ANNEX I

NUTRIENT LIMITATION AS A CAUSE OF GRADIENT-LIKE CARTILAGE MATRIX DISTRIBUTION

Iris A Otto
Gerwin Schreuder
Enric Mocholi
Paul Coffer
Jos Malda
Riccardo Levato

BACKGROUND

Cell survival as well as cellular function is highly dependent on the continuous supply of sufficient amounts of oxygen and other essential nutrients. Cartilage is a naturally avascular tissue and is therefore dependent on the diffusion of nutrients. In previous experiments, a gradient of cartilage-like tissue synthesis has been observed in cylindrical hydrogel constructs, with more matrix deposition in the peripheral areas. Nutrient limitation is likely to be responsible for this phenomenon. This experiment aimed at deducing the relative importance of oxygen and glucose metabolism in the ability of human auricular cartilage progenitor cells (AuCPC) to produce glycosaminoglycans (GAG). Oligomycin is a drug blocking mitochondrial respiration (oxygen metabolism), whereas 2-deoxy-D-glucose (2-DG) inhibits glycolysis (glucose metabolism).

MATERIALS & METHODS

Human adult AuCPCs ($n = 3$) were embedded in a 10 % w/v gelatin methacryloyl (gelMA) hydrogel at a density of 1.5×10^7 cells/mL and cast in cylindrical molds (diameter = 6 mm, height = 2 mm). Hydrogels were photo-crosslinked using UV-irradiation for 5 minutes ($\lambda = 365$ nm, $E = 6$ mW/cm^2, height = 3 cm). Cell-free samples were fabricated as controls. Samples were cultured for 14 days in chondrogenic differentiation medium with no drug supplement in the control group (group 1) or with the addition of either 1 µM oligomycin (group 2) or 8.2 mg/mL 2-deoxy-D-glucose (group 3). Cellular metabolic activity was evaluated through a resazurin assay. Biochemical composition was assessed through a dimethylmethyleneblue (DMMB) assay for the quantification of GAGs and a picogreen assay for the quantification of DNA. Safranin O staining on paraffin-embedded samples was used for histological analysis of GAG distribution. The penetration of glucose in gelMA hydrogels was assessed by incubating the samples for 3 hours in media containing 100 µM 2-NBDG (2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4yl)amino)-2-deoxyglucose) and visualizing the distribution of fluorescence. Analysis was performed using Graphpad Prism 8 and a statistical difference of $p < 0.05$ was considered significant.

RESULTS

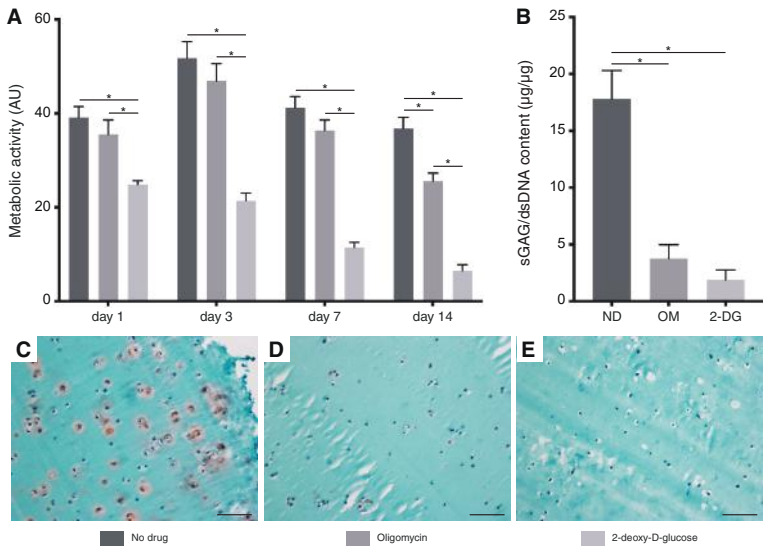

Figure 1. Blocking glycolysis or mitochondrial respiration in human AuCPCs results in decreased glycosaminoglycan production. Metabolic activity of AuCPCs in chondrogenic media over the course of 14 days, with no drug (ND) supplement or the addition of oligomycin (OM) or 2-deoxy-D-glucose (2-DG) (A). Quantification of GAG per DNA content after 14 days of culture in respective media (B). Safranin O staining visualizing the deposition of GAGs in hydrogels cultured in normal chondrogenic media (C) or supplemented with oligomycin (D) or 2-deoxy-D-glucose (E). Scale bars equal 100 µm. The asterisk (*) indicates a statistically significant difference ($p < 0.05$).

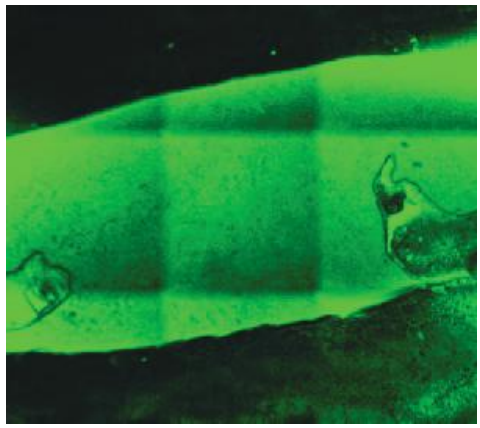

Figure 2. Glucose diffuses fully through 10 % w/v gelMA hydrogels. Fluorescent signal emitted by 2-NBDG was observed throughout the cylindrical hydrogel after 3 hours of incubation. Scale bar equals 200 µm.

CONCLUSION

The results from this experiment indicate that human AuCPCs are dependent on both oxygen and glucose for cellular health and the synthesis of GAGs. Metabolic activity of AuCPCs treated with 2-deoxy-D-glucose was significantly impaired from the start of culture. GAG deposition was markedly hampered by the inhibition of glycolysis as well as the blocking of mitochondrial respiration. As fluorescent glucose was able to penetrate the hydrogel fully, glucose is not likely to be the main limiting factor in the production of GAGs.

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ANNEX II

FUNCTIONALIZATION OF GELATIN METHACRYLOYL WITH MYOGLOBIN

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Tim BF Woodfield
Jos Malda
Riccardo Levato

The results in this annex have been included in the following publication:
Khoon S Lim, Florencia Abinzano, Paulina N Bernal,
Ane Albillos-Sanchez, Pau Atienza-Roca, Iris A Otto,
Tim BF Woodfield, Jos Malda, Riccardo Levato.

One-step crosslinking of a dual-functionalized bioink as
cell carrier and cartilage-binding glue for chondral regeneration.

Advanced Healthcare Materials (2020) e1901792.

BACKGROUND

Nutrient and oxygen limitation is largely responsible for cell death and central necrosis in large tissue-engineered constructs. In this experiment, we aimed to incorporate myoglobin into the hydrogel to serve as an oxygen reservoir in case of severe hypoxic circumstances. Gelatin methacryloyl (gelMA) is a highly suitable cell encapsulation platform that allows further modifications to improve its properties for tissue engineering and bioprinting. GelMA functionalized with tyramine moieties (gelMA-Tyr) allows for the covalent interaction between tyramine groups in the gel and tyrosine residues in proteins such as myoglobin.

MATERIALS & METHODS

GelMA was derivatized with tyramine moieties (gelMA-Tyr) to allow covalent bonding with tyramine residues. Both 10 % w/v gelMA and 10 % w/v gelMA-Tyr hydrogels were supplemented with 10 mg/mL equine muscle-derived deoxygenated myoglobin. The myoglobin-laden hydrogels were cast into cylindrical samples (3 replicates per timepoint; diameter = 6 mm, height = 2 mm) and subsequently crosslinked using a visible light-based photoinitiator system based on tris(2,2'-bipyridyl)ruthenium(II) chloride (Ru) and sodium persulfate (SPS). Samples were incubated at 37°C in PBS to assess protein release over a 48-hour timespan, with timepoints at 1, 15, 30 and 45 minutes and 1, 2, 3, 6, 24 and 48 hours. At each timepoint, hydrogel samples were collected for stereomicroscopy imaging and the media was analyzed with a UV-vis spectrometer to quantify the amount of released myoglobin over a wavelength range of λ = 360-460 nm. Myoglobin concentrations were derived from the peak absorbance value at 409 nm using a standard curve.

RESULTS

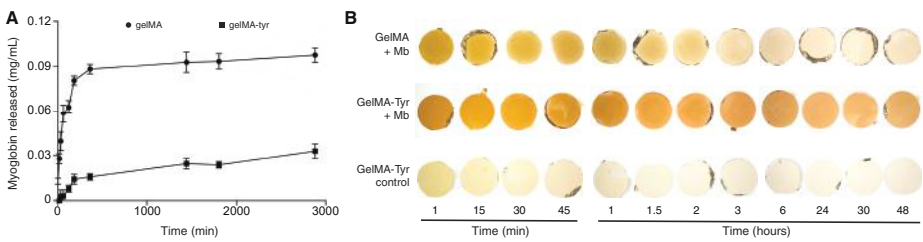

Figure 1. Incorporated myoglobin is retained in 10 % w/v gelMA-Tyr hydrogels. Concentrations of myoglobin that diffused out of hydrogel samples, as determined at the peak absorbance value of the myoglobin spectrum obtained through UV-vis spectroscopy (A). A t-test determined a statistically significant difference between the myoglobin concentrations in gelMA and gelMA-Tyr hydrogels after 48 hours of incubation. Qualitative assessment of deoxygenated myoglobin (dark brown) diffusivity out of hydrogel samples (B). Discoloration of the hydrogel indicated that freely-incorporated myoglobin in gelMA diffused out of the hydrogel. In contrast, gelMA-Tyr hydrogels retained the dark brown color, indicating immobilization of myoglobin.

CONCLUSION

Visual assessment of myoglobin binding in 10 % gelMA constructs demonstrated a gradual decrease in brown coloring, indicating the diffusion of myoglobin out of the hydrogels into the culture media. This was confirmed by UV-vis spectrometry, which displayed a steep increase in myoglobin concentration in the media in the first 6 hours of incubation after which a plateau phase is reached. In contrast, gelMA-Tyr constructs maintained the brown color over the entire 48-hour culture period and UV-vis spectrometry displayed constantly low levels of myoglobin in the culture media. These results show that deoxygenated myoglobin can be immobilized in gelMA-Tyr hydrogels, which holds promise for the provision of an extra supply of oxygen in large tissue-engineered cartilage constructs.

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LIST OF ABBREVIATIONS

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2-DG	2-deoxy-D-glucose
2-NBDG	2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4yl)amino)-2-deoxyglucose
3D	Three-dimensional
ACAN	Aggrecan
αMEM	Minimal essential medium – alpha modification
AM	Additive manufacturing
ANOVA	Analysis of variance
ATMP	Advanced Therapy Medical Product
ATP	Adenosine triphosphate
AuCH	Auricular chondrocyte
AuCPC	Auricular cartilage progenitor cell
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CAD	Computer-aided design
CAM	Computer-aided manufacturing
CBCT	Cone-beam computed tomography
COL	Collagen
COL1A1	Collagen type I
COL2A1	Collagen type II
COL10A1	Collagen type X
COMP	Cartilage oligomeric matrix protein
CPC	Cartilage progenitor cell
CSPC	Cartilage stem/progenitor cell
CT	Computed tomography
DAPI	4′,6-diamidino-2-phenylindole
dECM	Decellularized extracellular matrix
DICOM	Digital Imaging and Communication in Medicine
DMA	Dynamic mechanical analyzer
DMEM	Dulbecco's modified Eagle medium
DMMB	Dimethylmethylene blue
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
DSHB	Developmental Studies Hybridoma Bank
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FDM	Fused deposition modeling
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GelMA	Gelatin methacryloyl

GelMA-TYR	Gelatin methacryloyl functionalized with tyramine
HPRT1	Hypoxanthine phosphoribosyltransferase
HU	Hausfdorff units
ICP	Iterative closest point
Ig	Immunoglobulin
IGF	Insulin growth factor
IHC	Immunohistochemistry
ITS	Insulin-transferrin-selenium
MEW	Melt electrowriting
MSC	Mesenchymal stromal cell
mRNA	Messenger ribonucleic acid
µCT	Micro-computed tomography
NBF	Neutral-buffered formalin
OM	Oligomycin
PBS	Phosphate-buffered saline
PCL	Poly-ε-caprolactone
PCR	Polymerase chain reaction
PD	Population doublings
PEG	Poly(ethylene) glycol
PGA	Polyglycolic acid
PGLA	Poly(lactic-co-glycolic acid)
PLLA	Poly(l-lactic acid)
PSPC	Perichondrium stem/progenitor cell
RM	Regenerative medicine
RMS	Root mean square
RNA	Ribonucleic acid
RRI	Responsible Research and Innovation
RT-PCR	Reverse transcriptase polymerase chain reaction
Ru	Tris(2,2'-bipyridyl)ruthenium(II) chloride
RUNX2	Runt-related transcription factor 2
SD	Standard deviation
SEM	Standard error of the mean
sGAG	Sulphated glycosaminoglycan
SPS	Sodium persulfate
STL	Standard tesselation language
TE	Tissue engineering
TGF-β	Transforming growth factor beta
UV	Ultraviolet

NEDERLANDSE SAMENVATTING

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De menselijke oorschelp is een structuur aan beide zijden van de schedel. De unieke driedimensionale (3D) vorm ervan heeft als functie om binnenkomende geluidsgolven de gehoorgang in te leiden. Afwijkingen van de oorschelp kunnen worden veroorzaakt door fysiek trauma, verbranding of kanker, maar het komt ook voor dat kinderen worden geboren met een onderontwikkelde of zelfs afwezige oorschelp. Deze aangeboren aandoeningen heten respectievelijk microtie en anotie, en worden doorgaans chirurgisch behandeld met als doel het psychosociale welzijn van het kind te bevorderen.

De gouden standaard is de chirurgische reconstructie van de oorschelp met autoloog ribkraakbeen. Hierbij maakt de chirurg gebruik van delen van het ribkraakbeen van de patiënt om een geraamte te vormen dat gelijkend is aan de contouren van de normale oorschelp. Dit plaatst de chirurg vervolgens op de juiste plek onder de huid van de schedel. Deze operatie is één van de meest uitdagende technieken binnen de reconstructieve chirurgie. Er kunnen goede resultaten behaald worden in ervaren handen, maar het blijft een ingrijpende operatie met nadelen, zoals een litteken, mogelijk vervorming van de borstkas, pijn, en het risico op ongewenste vervorming van de gereconstrueerde oorschelp. Een andere optie is om gebruik te maken van een voorgevormd synthetisch implantaat van het poreuze materiaal polyethyleen. Dit materiaal is echter vele malen stugger dan ribkraakbeen en het risico bestaat dat het implantaat breekt of door de huid heen komt.

Regeneratieve geneeskunde kan uitkomst bieden met een elegant alternatief. Het is mogelijk om met speciale kweektechnieken nieuw weefsel te (re)genereren in het laboratorium met een combinatie van cellen, biomaterialen en stimulerende stoffen. Bioprint-technieken maken het mogelijk om deze ingrediënten op precieze locaties te plaatsen in 3D. Op deze manier kunnen specifieke weefsels en organen vormgegeven worden. Vervolgens worden de cellen in dit complexe ontwerp gestimuleerd om uit te groeien tot het bedoelde weefsel. Deze technologie biedt de mogelijkheid om kraakbeenconstructen te creëren die overeenkomen met de eigenschappen van de oorschelp. Echter moeten er nog fundamentele wetenschappelijke vragen worden behandeld om de kwaliteit en duurzaamheid van dergelijke constructen te waarborgen.

De centrale vragen in dit proefschrift zijn daarom: *Wat zijn de uitdagingen in de biotechnologische vervaardiging van een kraakbeenimplantaat voor oorreconstructie, en hoe kunnen deze uitdagingen overwonnen worden?* Hieruit kwamen de volgende doelen voort: het bepalen van een passend celtype voor het genereren van elastisch kraakbeen (**Deel I**), het fabriceren van een versterkt oorimplantaat voor verbetering van vorm en stabiliteit (**Deel II**), en het beoordelen van

ethische factoren in bioprint-onderzoek en het betrekken van maatschappelijke belanghebbenden voor een meer verantwoordelijk onderzoeksproces (**Deel III**).

In **Hoofdstuk 2** worden de belangrijkste uitdagingen in het genereren van oorkraakbeen uitgediept, waaronder het verkrijgen van voldoende kraakbeen-producerende cellen, het creëren en behouden van de complexe vorm van de oorschelp, en de voorziening van een ondersteunende micro-omgeving. Deze uitdagingen vormen de basis van het onderzoek in dit proefschrift.

DEEL I – CELLEN: BEPALING VAN EEN PASSEND CELTYPE VOOR REGENERATIE VAN OORKRAAKBEEN

De eerste uitdaging is om voldoende autologe kraakbeen-producerende cellen te verkrijgen om een weefsel ter grootte van de menselijke oorschelp te genereren. In het onderzoek naar kraakbeenregeneratie worden veelal chondrocyten, de cellen uit het kraakbeen, gebruikt. Echter zijn er maar kleine hoeveelheden van deze cellen verkrijgbaar uit het weefsel en moeten ze veelvuldig worden vermeerderd, wat leidt tot een sterk verminderde kwaliteit van het weefsel dat deze cellen uiteindelijk zullen produceren. Stamcellen uit het beenmerg kunnen wel blijven delen zonder dat ze hun regeneratieve capaciteiten verliezen, maar deze cellen hebben de voorkeur om bot te regenereren in plaats van kraakbeen. In **Hoofdstuk 3** hebben we een nieuwe celsoort geïdentificeerd in het oorkraakbeen van paarden: de auriculaire kraakbeen progenitor cel (AuCPC). Deze cellen hebben stamcel-achtige kwaliteiten en kunnen gemakkelijk vermeerderd worden. Tegelijkertijd zorgt hun afkomst uit het oorkraakbeen ervoor dat ze een voorkeur hebben voor het genereren van kraakbeen. Na het kweken van AuCPCs in een hydrogel bleek inderdaad dat deze cellen kraakbeen van goede kwaliteit kunnen produceren en dat op genetisch niveau geen blijk was van differentiatie richting bot. De aanwezigheid van deze cellen in menselijk oorkraakbeen hebben we bevestigd in **Hoofdstuk 4**. Het oorkraakbeen van zowel volwassenen als kinderen, alsook het rudimentaire stukje oorkraakbeen van microtie patiënten, bevat kraakbeen-regenererende AuCPCs. Middels groeiexperimenten hebben we berekend dat uit een biopt van het normale oor voldoende AuCPCs verkregen kunnen worden voor de vervaardiging van een menselijk oorimplantaat. Ook de menselijke AuCPCs bleken in staat om kraakbeen-specifiek weefsel te produceren in een 3D hydrogel. Deze resultaten suggereren dat AuCPCs een belangrijke oplossing kunnen bieden voor de cellulaire uitdagingen in kraakbeenregeneratie, en kunnen bijdragen aan de vertaling van de weefselkweektechnologie naar klinische toepassing.

DEEL II – FABRICATIE: OP ZOEK NAAR EEN DUURZAAM OORIMPLANTAAT

Een volgende uitdaging is om de unieke vorm van het oor te fabriceren en om die te behouden na implantatie. De onderontwikkelde oorschelp in microtie patiënten gaat meestal gepaard met een flink kraakbeentekort, waardoor er voor reconstructie ook te weinig huid beschikbaar is om het implantaat te bedekken. In **Hoofdstuk 5** presenteerden we een beeldvormingsmethode om op een betrouwbare manier de oppervlakte van het oor te berekenen. Met deze methode stelden we ook vast dat er gemiddeld een huidtekort van meer dan 50% is aan de zijde van de microtie in vergelijking met de normale oorschelp aan de andere zijde. De samentrekkende krachten van de huid zorgen voor een belangrijke uitdaging voor het onderliggende implantaat. Bij de stugge ribkraakbeen- en synthetische implantaten moet men vooral bedacht zijn op het doorbreken van de huid, terwijl de literatuur over gekweekte weefsels juist krimp en vervorming van oorvormige implantaten meldt. Daarom is het essentieel om de hydrogel die als celdrager voor het kweken van weefsel wordt gebruikt mechanisch te verstevigen. In **Hoofdstuk 6** verweefden we daarom middels bioprint-technieken synthetische polymeervezels in het hydrogel construct om als ondersteunende steiger te fungeren. Hiermee werden de constructen versterkt tot een vergelijkbaar niveau als natuurlijk oorkraakbeen. Ook stelden we vast dat de levensvatbaarheid en het regeneratief vermogen van AuCPCs niet negatief beïnvloed werden door het printproces of de vezelversteviging. Met een digitaal ontwerp gebaseerd op het geraamte dat de chirurg boetseert tijdens de reconstructie, hebben we vervolgens met dezelfde bioprint-technieken concept-oorschelpen gefabriceerd. Na een dynamisch kweekproces hebben we met geavanceerde beeldvormingstechnieken bepaald dat de vorm en grootte van de constructen tijdens deze periode behouden waren, en dat de aanwezige cellen rijkelijk nieuw kraakbeenweefsel hadden geproduceerd in de gehele oorvormige constructen. De methode die wij gebruikt hebben voor het fabriceren van een oorconstruct op ware grootte – de combinatie van menselijke AuCPCs, een ondersteunende hydrogel, en verstevigende vezels die zijn geplaatst middels bioprint-technieken – is daarom een interessante strategie om verder te ontwikkelen.

DEEL III – SAMENLEVING: BIOPRINT-ONDERZOEK IN EEN ETHISCHE CONTEXT

De ontwikkeling van gekweekte weefsels en bioprint-technieken leidt ook tot ethische vragen. Want wat zal de invloed van deze innovatieve technieken zijn op de samenleving? In parallel met een ethische analyse kan wetenschappelijk onderzoek anticiperen op deze effecten en een verantwoord onderzoeksproces stimuleren. Daarom hebben we in **Hoofdstuk 7** onderzocht welke ethische factoren een rol spelen in het domein van bioprinten. Bij het gebruik van menselijke cellen moet worden nagedacht over hoe deze cellen op een geschikte wijze kunnen worden verkregen,

opgeslagen en gebruikt. Voor proeven met geprinte weefsels in mensen moeten de bestaande ethische en wettelijke kaders verfijnd worden specifiek voor deze categorie technieken. Ook een inventarisatie van de verwachtingen en zorgen van de samenleving is van belang. Zonder betrokkenheid van maatschappelijke belanghebbenden, zoals patiënten of gebruikers, kunnen onderzoeksprocessen en resultaten verkeerd worden afgestemd op maatschappelijke waarden en behoeften. Verantwoorde innovatie omvat de betrokkenheid van verscheidene maatschappelijke belanghebbenden om zo effectieve bruggen te bouwen tussen onderzoek, kliniek en de samenleving. Om de stap te zetten naar het verbeteren van de betrokkenheid van patiënten in het onderzoeksproces, hebben we in **Hoofdstuk 8** de houding van ouders van kinderen met microtie onderzocht ten opzichte van de nieuwe technieken die worden ontwikkeld voor reconstructie van de oorschelp. Hieruit bleek dat de meerderheid van de ouders erg positief is over het kweken van nieuw kraakbeen in het laboratorium en het bioprinten van oorimplantaten. Echter kwam ook naar voren dat ouders terughoudend zijn om hun kind als een van de eersten mee te laten doen aan een eventuele klinische trial. Zij gaven aan liever af te wachten tot de technieken uitgebreider getest zijn en er meer informatie beschikbaar is over de risico's. Deze resultaten onderstrepen het belang van een actieve rol van patiënten en andere maatschappelijke belanghebbenden bij het ontwikkelen van wetenschappelijk onderzoek.

Hoofdstuk 9 vat de resultaten van bovenstaand onderzoek samen en plaatst deze in een wetenschappelijke en maatschappelijke context. Het werk in dit proefschrift heeft geleid tot de introductie van een nieuw veelbelovend celtype voor de regeneratie van oorkraakbeen, een strategie voor het fabriceren van oorconstructen die duurzaam in vorm en grootte zijn, en een aanbeveling voor het opnemen van ethische analyses in het onderzoeksproces en het betrekken van maatschappelijke belanghebbenden zoals patiënten.

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DANKWOORD / ACKNOWLEDGEMENTS

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Eleanor Roosevelt

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LIST OF PUBLICATIONS

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THIS THESIS IS BASED UPON THE FOLLOWING PUBLICATIONS

IA Otto, PE Capendale, JP Garcia, M de Ruijter, RFM van Doremalen, M Castilho, T Lawson, MW Grinstaff, CC Breugem, M Kon, R Levato, J Malda. Biofabrication of a shape-stable auricular structure for the reconstruction of ear deformities. *Submitted.*

IA Otto, PN Bernal, M Rikkers, M Kon, CC Breugem, J Malda, R Levato. Fibronectin-adhering progenitor cells reside in human adult, pediatric and microtia auricular cartilage and harbour a potential for regenerative ear reconstruction. *Submitted.*

IA Otto, A Simone, J Malda, M Kon, CC Breugem. Engaging stakeholders in bioprinting research: Views and concerns of microtia patients' parents on bioprinted auricular cartilage. *Journal of Plastic, Reconstructive and Aesthetic Surgery* 2020.

IA Otto, R Levato, WR Webb, IM Khan, CC Breugem, J Malda. Progenitor cells in auricular cartilage demonstrate cartilage-forming capacity in 3D hydrogel culture. *European Cells & Materials* 2018; 35: 132–150.

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IA Otto, J Malda. 3D-bioprinten. In: Biowetenschappen en maatschappij. *Stamcellen: Wetenschap, werkelijkheid en fictie*, 2016; 2: 35–38.

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CURRICULUM VITAE

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Iris Otto was born on the 14th of July 1988 in Utrecht, The Netherlands, to Evert Otto and Marian Overduin. She grew up in Nijkerkerveen together with her brother Florian and followed a bilingual secondary education at 't Atrium in Amersfoort. After obtaining her diploma in 2006, she attended University College Utrecht (UCU), where she pursued a pre-medical track as well as a minor in psychology and development studies. During her bachelors, Iris studied a semester at Macquarie University in Sydney (Australia), participated in the UCU in Africa development program and interned at Mainyoto Pastoralists Integrated Development Organization (MPIDO) in Kenya to return research results on health and education to local communities. She graduated *cum laude* from UCU in 2009. The following year she worked as a physician assistant at a general practice and as a research assistant at the Department of Surgery of the University Medical Center Utrecht (UMCU; The Netherlands). She was also appointed Vice Chair of the University College Alumni Association (UCAA) board and was editor of UCAA's alumni magazine Post. In 2010 she continued her education at Utrecht University (UU) to obtain her MD qualification through the Selective Utrecht Medical Master (SUMMA). She did her clinical rotations at Gelre Ziekenhuizen in Apeldoorn, Medisch Centrum Soesterberg, and Academisch Ziekenhuis Paramaribo (Suriname). She also participated in the UU Honors program Young Leaders League.

Under the joint supervision of prof.dr.ir. Jos Malda (Department of Orthopaedics, UMCU) and prof.dr. Moshe Kon (Department of Plastic, Reconstructive and Hand Surgery, UMCU), Iris started a research internship on biofabrication of ear cartilage in 2013. She gratefully accepted the opportunity to conduct part of her research at the Laboratory for Musculoskeletal Tissue Engineering at Massachusetts General Hospital in Boston (USA) under the supervison of prof.dr. Mark Randolph and dr. Xing Zhao. After graduating from medical school in 2015, Iris was awarded a PhD Fellowship in Regenerative Medicine from the Dutch Research Council (NWO) that allowed her to continue her research with prof.dr.ir. Jos Malda, prof.dr. Moshe Kon, dr. Corstiaan Breugem and dr. Riccardo Levato. The focus of her work was on cell-based cartilage tissue engineering, fabrication of durable auricular shapes, and the ethical aspects of biofabrication technologies.

The work described in this thesis was presented at various national and international conferences. In 2017, Iris was awarded the Burt Brent Prize for best presentation at the triennial congress of the International Society for Auricular Reconstruction (ISAR) in Beijing (China), as well as the Best Chair Award at the annual Netherlands Society for Biomaterials and Tissue Engineering (NBTE) conference. She also participated in various public engagement activities to inform and enthuse the public about biofabrication research. In 2018, Iris won the UU Breaking Science pitch competition, explaining her research to a lay audience. Together with dr. Corstiaan Breugem she appeared in the national television show *TopDoks*, where she was interviewed on how 3D bioprinting can be used for future ear reconstruction. During her appointment, Iris also developed an online elective course for the UU Graduate School of Life Sciences, entitled 'Fundamentals of Biofabrication'. In 2019, she obtained her University Teaching Qualification from Utrecht University.

Iris is now on a mission to improve societal health standards through awareness, prevention and lifestyle interventions. In 2020 she founded Gezond Kompas, an online educative platform on sustainable health.