

Bioengineering Human Scaffold-free Cartilage Constructs Using Paediatric Auricular Tissue

by

Pedram Akbari

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Abstract

Currently, patients with external ear deformities rely on hyaline cartilage grafts or silicone prosthesis to provide them with adequate external ear reconstruction. Scaffold-free auricular tissue engineering provides a means of creating patient specific cartilage constructs solely using their own cells, ensuring biocompatibility, long term stability, and reduced morbidity. Here, we have developed a simple method for generating scaffold-free auricular constructs using paediatric auricular cartilage remnants. High cell density micromass cultures treated with chondrogenic medium for prolonged periods result in mechanically stable cartilage constructs resembling native paediatric auricular cartilage mechanically, histologically, immunohistochemically, and biochemically.

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Chapter 1

The Human External Ear

1.1 Introduction

The external ear, often referred to as the auricle or the pinna, is physiologically responsible for collecting sound waves and directing them towards the eardrum (Edgar, 1974; Rosowski, 1994). This protects the fragile eardrum by providing a long and narrow entrance to the auditory center (Edgar, 1974). It also provides acoustical gain for lower frequency sounds and its presence and function are crucial for the special perception of sound (Edgar, 1974). However, besides its physiological function, it is also an important facial structure that prominently contributes to an individual's overall facial appearance, and thus self-esteem (Reiffel, et al., 2013). The external ear is composed of a thin layer of elastic cartilage surrounded by vasculature and an epithelium integument, and although its composition consists of only a handful of different cell types, its three dimensional (3-D) anatomical complexity and elastic cartilaginous core are what make this anatomical feature unique, and challenging to repair.

1.2 External Ear Development

Early developmental observations of the external ear have dated as far back as the early 1880s, when Wilhelm His first described the presence of 6 distinct buds that protrude from the branchial arches in the human embryo (His, 1882). Three hillocks are found on the first branchial arch, and three found on the adjacent, second branchial arch, and together they are commonly known as the Hillocks of His (Cox, Camci, Vora, Luquetti, & Turner, 2014). The 6 structures, first visible six weeks into embryogenesis, fuse together and give rise to the complete external ear by the 8th week of embryogenesis (His, 1885). His proposed a specific pattern of development which linked each hillock to a specific auricular segment, however, throughout subsequent literature there has been controversy regarding which hillock gives rise to which auricular structure, which was summarized in a review (Streeter, 1922). The current general consensus is depicted in **Figure 1-1**.

The pinna consists of a number of different anatomical features fused together, with each structure having its own unique 3-dimensional shape. The complexity of each individual auricular feature, in combination with its adjacent complex features together yields the external ear as a very individualistic component of the human body. This spatial complexity is one of the reasons why reconstruction of the external ear has been deemed extremely difficult (Nayyer, et al., 2012).

1.3 Molecular Development

In early development, prior to cartilage formation, rapidly proliferating and loosely arranged mesenchymal stem cells receive chondrogenic stimuli, such as TGF- β , BMP, and FGF signalling factors, causing them to undergo condensation, forming precartilaginous nodules as depicted in **Figure 1-2** (Matta & Zakany, 2013; Smits, et al., 2001). Upon further differentiation, these nodules then become cartilaginous in their cores, which then go on to form the skeleton. Although this developmental scheme is commonly identified in bone/hyaline cartilage formation, it is believed to be conserved throughout early elastic cartilage development as well.

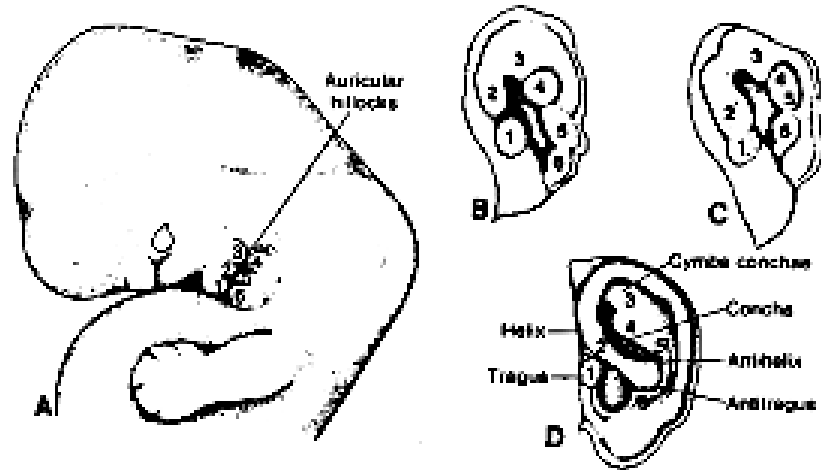


Figure 1-1. Development of the human external ear.

Figure is taken from Wright (1997), which highlights the fusion of the hillocks of His, rendering the recognizable developed external ear.

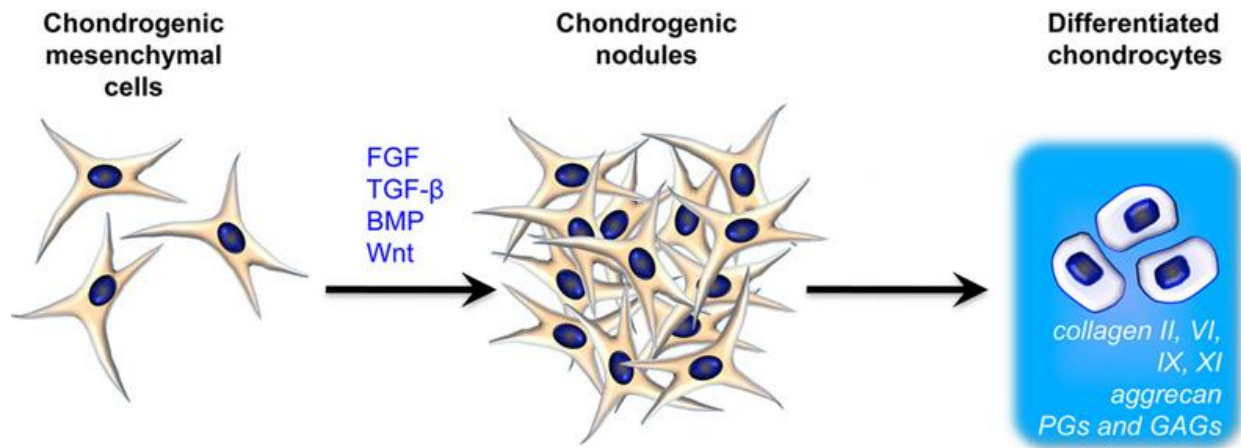


Figure 1-2. Mesenchymal Condensation required for early chondrogenesis during limb development

Taken from Matta (2013), Mesenchymal condensation occurs upon receiving chondrogenic stimuli, initiating chondrogenesis.

1.4 Auricular Landmarks

The developed human pinna is an extremely complex 3-D structure containing numerous individual anatomical structures. The major anatomical features of the pinna and their corresponding Hillocks of His are depicted in **Figure 1-3** (Gray, 1918). In summary, the external ear consists of two closely positioned bulges, with the superior anterior bulge being called the tragus, and its counterpart being antitragus. Additionally, an outer ridge called the helix runs from above the tragus towards the antihelix in an oval shape in the shape of an oval consisting of ascending, horizontal, and descending helix components, the superior posterior anatomical position of the tragus towards the antitragus. An inner ridge, referred to as the antihelix, runs parallel with the descending portion of the helix, ultimately connecting to the antitragus. The antihelix also consists of two crura, which run perpendicular to the rising helix and the horizontal helix. Finally, at the base center of the external ear, which also connects multiple external ear structures as well as connecting the external ear to the skull is the conchal bowl (Alvord & Farmer, 1997). These structures are unique amongst individuals thus the individual anatomical complexities accumulate to form an overall structure of momentous complexity.

1.5 Human External Ear Tissue Composition

The external ear consists of an elastic cartilage core which provides the pinna with its framework integrity. This elastic cartilaginous core consists of a cartilaginous component (chondrium) enclosed by a fibrous layer called the perichondrium. This core structure is then surrounded by soft connective tissue, small amounts of subcutaneous fat, vasculature, sebaceous and sweat glands, and, hair (Oztürkcan & Oztürkcan, 2014). The cross section of the pinna depicting the different components is shown in **Figure 1-4**.

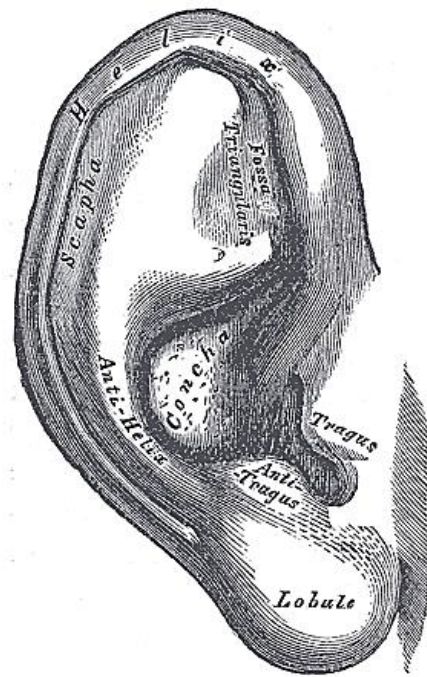


Figure 1-3. Anatomy of the human external ear.

Figure taken from Gray (1918), the anatomy of the external ear.

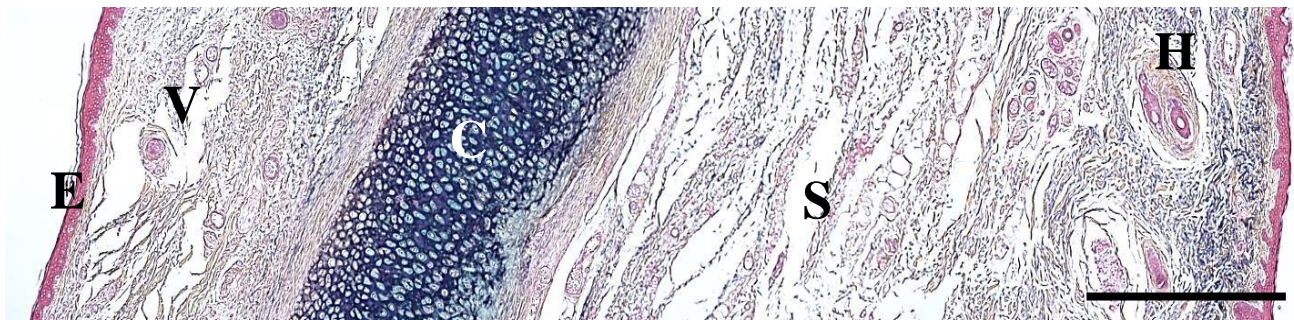


Figure 1-4. A cross section of the human external ear (Movat Stain), highlighting all important structures. E – Epidermis.

V – Vasculature, C – Elastic Cartilage, S - Soft tissue, H - Hair follicle/ sebaceous gland. Scale bar: 1 mm.

Anatomical directionality: Lateral - Medial

1.6 Cartilage

Cartilage is mechanically strong connective tissue found in many different locations in the body (Wachsmuth, Söder, Fan, Finger, & Aigner, 2006). It is mostly composed of cartilage specific extracellular matrix (ECM), and its resident cells called chondrocytes. There are three main types of cartilage, elastic cartilage, hyaline cartilage, and fibrocartilage. These different types of cartilage are each specialized and thus specific to different anatomical locations throughout the body, however, despite many similarities, they are easily distinguished based on ECM content and overall architecture. Commonly, the major components of cartilage are: collagen type II, chondroitin sulphate/aggrecan glycosaminoglycans (GAGs), with addition to collagen type X for hypertrophic cartilage (Shen, 2005), elastin for elastic cartilage (Reiffel, et al., 2013), and collagen type I for fibrocartilage (Kambic & McDevitt, 2005).

1.7 Elastic Cartilage

Elastic cartilage is a type of dense irregular connective tissue most commonly found in the external ear, the middle ear (Eustachian tube), and the epiglottis, and offers the flexibility and strength required (Parsons, 1998) for such structures to maintain their function. The presence of elastin in this type of cartilage distinguishes it from the other types of cartilage: hyaline and fibrocartilage. Hyaline and fibro cartilage are mechanically strong and are able to bare heavy loads, whereas elastic cartilage is mainly utilized for its flexibility (Hinderer, et al., 2015). Similar to hyaline cartilage, elastic cartilage has a high composition of collagen II, low levels of collagen type I, and very high levels of sulphated Glycosaminoglycans (GAGs), however unlike hyaline nor fibrocartilage, elastin cartilage exhibits the abundant presence of functional elastic fibers, providing the tissue with flexibility that is not observed in the other two types of cartilage.

1.7.1 Elastin

Elastic fibers are composed of two components: an amorphous elastin component and an elastic microfibril component (Kielty, Sherratt, & Shuttleworth, Elastic fibres., 2002). Both components are rich in simple amino acids such as glycine, alanine, valine, and proline, which together provide the protein with extensive conformational elasticity in the form of elastic coils (Robb,

Wachi, Schaub, Mecham, & Davis, 1999). The amorphous component is made up of polymerized tropoelastin (the soluble precursor protein to mature elastic fibers), and the microfibril consists of several types of proteins such as fibulins and fibrillins which act as a scaffold to organize the deposition of amorphous elastin (Kielty, Sherratt, & Shuttleworth, 2002). Tropoelastin, encoded by the gene (*ELN*), is a 60-70 kDa soluble, non-glycosylated protein, that undergoes posttranslational modifications, transportation to the extracellular, and crosslinking by lysyl oxidase to form functional mature elastic fibers (Hinderer, et al., 2015). Elastic fibers are responsible for providing elasticity and strength to numerous types of tissue throughout the human body such as the lungs, heart, arteries, skin, and auricular cartilage. Mutations in the *ELN* gene can result in abnormal functioning of the tissues or organ that relies on the mechanical properties of elastic fibers. For example: Cutis laxa is a skin disorder in which patients exhibit loose skin, and it has been linked to *ELN* mutations and deletions (Hbib, et al., 2015). Another example: Supravalvular aortic stenosis, narrowing of the aorta is due to significantly reduced levels of elastic fibers arising from deleterious *ELN* mutations and insufficiencies (Nassar, Pushparajah, Bell, & Austin, 2015). Due to the severe phenotype of *ELN* dysregulation, elastogenesis has been the subject of intensive research. Besides elastic cartilage, elastin is also importantly found in the skin and the aorta, in which it confers resilience allowing stretched skin to return to its native form, and the aorta to be able to accommodate the high pressured blood leaving the left ventricle (Kielty, Stephan, Sherratt, Williamson, & Shuttleworth, 2007).

1.8 Microtia

Microtia is a congenital deformity encompassing a range of phenotypes associated with the underdevelopment of the pinna; affecting approximately 1 in 000 live births (Yanaga, Imai, Fujimoto, & Yanaga, 2009). Microtia can affect only one side (unilateral, > 75%), or affect both sides (bilateral), and can either be seen as part of a syndrome including Treacher Collins and other syndromes within the facio-auriculo-vertebral spectrum (Goldenhar's syndrome, oculoauricular vertebral dysplasia and hemifacial microsomia), or as seen in many cases, in isolation (Muñoz-Pedroza & Arenas-Sordo, 2013). It may be that Goldenhar's syndrome, hemifacial microsomia, oculoauriculovertebral dysplasia, and microtia are simply variants of the same or similar entities (Luquetti, Heike, Hing, Cunningham, & Cox, 2012). Acquired microtia

can also occur following trauma, including burn and dog bites. Cases that present bilateral microtia, are more likely to have additional associated anomalies. Even though microtia is mostly identified as an external ear anomaly, a large proportion of patients with microtia (>90%) exhibit hearing loss on the site of the microtic ear (Luquetti, Heike, Hing, Cunningham, & Cox, 2012). This may be due to developmental anomalies in the middle or inner ear that sometimes accompany external ear deformities. A study in which 149 microtia cases were studied, positive inbreeding was identified in 14 patients, and a family history of the deformity was observed in 37 patients. This pattern of penetrance suggests an autosomal dominant inheritance pattern (Muñoz-Pedroza & Arenas-Sordo, 2013). Unfortunately, even minor cases can significantly impact a child's self-esteem and self-perception while growing up, heavily affecting their psychosocial functioning (Johns, Lucash, Im, & Lewin, 2015). Classification schemes are further complicated if the status of the external auditory canal (EAC) and middle-ear contents are included with the presence or absence of an associated syndrome (Luquetti, Heike, Hing, Cunningham, & Cox, 2012). As a result, classification of microtia is usually simplified by evaluating the external auricular deformities alone. They are as follows.

- 1) Grade I - Slight incompleteness of the pinna development. All major structures are present to some degree, and reconstruction does not require additional tissue.
- 2) Grade II - Partial incompleteness of the pinna development. There is a deficiency of tissue, and surgical correction requires the addition of cartilage and skin. Mini-ear and severe cup-ear deformities are included in this category.
- 3) Grade III - Only the presence of a peanut-like structure, common ear canal closure. Deformities have few or no recognizable landmarks of the auricle, although the lobule is usually present and positioned anteriorly.
- 4) Grade IV – Commonly referred to as Anotia, it entails the complete absence of the entire ear. (Johns, Lucash, Im, & Lewin, 2015)

Grade three is the most prevalent form of microtia. Because of the extreme underdevelopment of the external ear, and the closing of the ear canal that is associated with it, medical intervention is required.



Figure 1-5. Different grades of Microtia.

Figure taken from <http://earcommunity.com/microtiaatresia/>

1.9 External Ear Trauma & Amputations

There are other cases besides microtia which call for auricular reconstruction; such as amputations due to trauma, thermal damage and cancer (Stewart M. G., 2005; Turpin, Altman, Cruz, & Achauer, 1988; Ibrahim & Salem, 2008; Norman, Cracchiolo, Allen, & Soliman, 2015). The pinna is a protruding anatomical structure, and because of this, it is more susceptible to accidental damage (Lee & Sperling, 1996). Because of this, in many motorized vehicle accidents, there are instances in which the pinnae are amputated (Templer & Renner, 1990). Because the amputated tissue is still normal, it is commonly used for the repair surgery, however, there are many complications the surgeon must consider, such as the amount of arterial flow to the amputated tissue, because that is required for the tissue to survive (Templer & Renner, 1990). Another consideration is whether or not the amputated tissue has intact skin or not (Saad Ibrahim, Zidan, & Madani, 2008). If the amputated tissue is lacking skin, then flaps taken from the scalp may have to be used, further complicating the procedure (Saad Ibrahim, Zidan, & Madani, 2008). More common amputations of the pinna are due to tissue removal due to cancerous lesions (Kadakia, Saman, Gordin, Marra, & Ducic, 2015). Approximately 85,000 people were diagnosed with cancer in 2014 in Canada, and the equivalent of that for the U.S.A is roughly 1,000,000 people (Canadian Cancer Society, 2014; Siegel, 2014). 10% of these cases involved the ear, and nearly a third of all these cases showed invasion of the cartilage of the external ear, resulting in amputation of the ear (Ahmad & Das Gupta, 2001; Cox, Jones, & MacKie, 1987).

1.10 Current Treatment Options

Regardless of severity, microtia deformities can have a significant impact on a child and his or her self-perception (Johns, Lucash, Im, & Lewin, 2015). In fact, a study investigating the psychological profiling of congenital microtia patients identified that over 20.2 % of patients suffered from depression, 36.6% suffered from interpersonal sensitivity and social difficulties, and 26.3 % suffered from hostility and/or aggression (Jiamei, et al., 2008). As such, many children may benefit from procedures that aim to reconstruct their microtic ear. Current treatments include 1) a two-step surgical procedure involving a) fabrication of autologous costal

cartilage and b) its implantation, framework elevation, and its projection at the auricular site (Nagata, 1993; Tanzer, 1959; Brent, 1999), and 2) the use of prosthetics, either adhesive or implant retained (Thorne, et al., 2001; Demir, Malkoc, Ozturk, & Tosun, 2010; Federspil, 2010; Butler, Gion, & Rapini, 2000). Unfortunately, the surgical procedure is unusually difficult. Microtia remains one of the greatest challenges for reconstructive surgeons (Shieh, Terada, & Vacanti, 2004). Auricular reconstruction of the microtia deformity is a complex and labour-intensive process that requires a great deal of preoperative planning, surgical skill and artistry, and is accompanied by numerous severe complications and thoracic wall morbidities, in the case of costal cartilage harvest, which may compromise the function of the thoracic wall (Thomson, Kim, & Ein, 1995).

While children with microtia are initially seen and evaluated in early childhood, observation is recommended until the child is at least 6-8 years old, since at that age the external is 85% of its full grown size (DellaCroce, Green, & Aguilar, 2001). This is to ensure sufficient development of costal cartilage as well as the contralateral normal ear, optimizing the earliest possible age of successful surgery. Reconstruction using the standard rib-grafting technique requires two to four stages, each separated by 3 to 4 months (Chauhan & Guruprasad, 2012), beginning when the patient is approximately 6 to 8 years old.

1.10.1 Complications

Surgical complications include infection, bleeding, scarring, graft loss, displacement, resorption, asymmetry, and pneumothorax during harvest of the costal cartilage (Brent, 1999). The possibility of a less-than-optimal aesthetic result must be understood and the expectations of the patient, parents, and surgeon must be realistic and clearly defined. Even in the hands of very experienced surgeons and in the absence of complications, the ideal reconstructive results always fall short of the pliability, discrete contours and definition of the non-microtic auricle (Romo, Presti, & Yalamanchili, 2006).

1.10.2 Regenerative Medicine

Having a means of obtaining cartilage without reliance on these large grafts would constitute a major accomplishment in the practice of medicine, and auricular reconstruction surgeries.

Prosthetic implants still require the surgical removal of the native underdeveloped ear, and their installation prosthetic implants are susceptible to infections and can fail with small trauma whilst lacking human equivalent temperature, texture, and skin tone (Tollefson, 2006). Bioengineering a dynamically remodelling auricular construct using the patient's own cells would ensure biocompatibility, developmental plasticity, and a simpler surgical procedure. This would constitute significant advancement in medical practice which can greatly improve the quality of life for children suffering from this deformity.

Chapter 2

Background: Auricular Tissue Engineering

2.1 Introduction

Carving out patient specific auricular structures with delicate curvatures, from a hyaline cartilage that is already structurally limited due to its bioavailability, in the short amount of time that an invasive surgery permits, makes external ear reconstruction an extremely difficult surgical task (Ciorba & Martini, 2006). The purpose of auricular tissue engineering is to provide a means of using small biopsies to generate anatomically specific cartilage constructs in a patient specific manner *ex vivo*, such that the surgeon can simply implant the construct subcutaneously, in a rather trivial surgical procedure. This method would eliminate the requirement of large tissue harvests reducing donor site morbidities, all while enhancing the patient specificity of auricular grafts to be received, both at the cellular, mechanical, and anatomical level.

Another benefit of using tissue engineering to address the demand of auricular tissue is that the tissue construct used for reconstruction does not have to be of hyaline cartilage, unlike current methods using costal cartilage grafts for auricular repair. Currently, a significant amount of effort of scientists in the field is being focused on generating auricular tissue constructs using auricular chondrocytes, making the tissue graft similar to that of its native counterpart (Ciorba & Martini, 2006).

However, to be able to bioengineer such auricular tissue constructs for patients, there are many parameters that need to be examined and carefully evaluated. Several parameters have so far been specified, however, many more need to be evaluated.

2.2 Previous Work

2.2.1 Cell Sources for Auricular Tissue Engineering

To ensure biocompatibility and eliminated immunogenic responses, patient specific cell sources capable of auricular cartilage tissue regeneration need to be examined. This is very important, as both parameters of tissue harvest and the quality of auricular tissue generated by harvested tissue need to be carefully examined.

To be able to generate a clinically valuable tissue construct from biopsies, there needs to be an *in vitro* expansion phase, in which cells isolated from the small biopsy would replicate and ultimately yield a cell number that makes it feasible for patients construct formation. This step seems to be the most intrinsic and fundamental challenge within the field, as it is commonly observed that *in vitro* cellular expansion reduces the elastic cartilage forming capabilities of these cells (Schulze-Tanzil, 2009; Ma, et al., 2013; Karlsen, Shahdadfar, & Brinchmann, 2011).

2.2.1.1 Chondrocytes

Chondrocytes, the only cells found in healthy cartilage, are the most commonly used and studied cell source for auricular cartilage tissue engineering. Surrounding the cartilage is a fibrous layer called the perichondrium, which many believe to harbours populations that are cartilage precursor cells due to their higher rate of proliferation, multilineage differentiation potential, and expression of stem cell marker CD44 and CD90 (Kobayashi, et al., 2011; Sart, Schneider, & Agathos, 2009). To date, most studies harvesting chondrocytes from cartilage tissue do not specify the separation of the perichondrium from the chondrium when they digest the tissue and isolate cells, therefore cell populations commonly isolated likely contained these identified precursor cells.

Even though auricular chondrocytes are specialized cells, they are not terminally differentiated and are able to undergo rather slow expansion (Saadeh, et al., 1999). However, chondrocyte expansion dedifferentiates the chondrocytes into a fibroblast like state in which the chondrocytes lose their chondrogenic behaviour and expression profile of chondrocyte specific markers such as sox 9, collagen type II, aggrecan core protein, and Elastin, and adapt a fibroblastic

morphology, with upregulation of fibroblast markers such as collagen type I (Ishak, et al., 2011). Due to this dedifferentiation phenomenon, a lot of effort is being focused on identifying methods and factors to help reduce chondrocyte dedifferentiation and enhancing their re-differentiation potential (Xu, et al., 2011; Liu, et al., 2014)

2.2.1.2 Mesenchymal Stem Cells

2.2.1.2.1 Bone Marrow Derived Mesenchymal Stem Cells

One of the most commonly used sources of mesenchymal stem cells for cartilage tissue engineering is the bone marrow. Bone marrow derived mesenchymal stem cells (BM-MSCs) are abundant, easy to obtain, and highly proliferative, and are a promising source of cells for cartilage tissue engineering purposes (Saha, Kirkham, Wood, Curran, & Yang, 2010).

Studies comparing the chondrogenic potential of chondrocytes and BM-MSCs showed that cultured chondrocytes exhibit much higher levels of collagen type II and type X mRNA, quantified via RT-qPCR (Chiang, et al., 2011) (Saha, Kirkham, Wood, Curran, & Yang, 2010). These studies also evaluated the *in vitro* chondrogenesis potential of the two and identified that constructs generated using chondrocytes exhibited a better cartilage like ECM than that of BM-MSCs population. Other studies involving the chondrogenic differentiation of BM-MSCs, reported osteogenesis, vascularization, shrinkage, and the hollow phenomenon (Xue, Qi, Zhou, & Liu, 2013) that would all be very detrimental to auricular cartilage construct formation.

A probable explanation for these observations is that BM-MSCs are capable of multilineage differentiation, and if not carefully guided through chondrogenesis, then the cell population will go down a different path. Therefore, optimization of culture conditions maybe what is required for BM-MSCs to be able to produce better cartilage, and thus there are currently large ongoing efforts (Xue, Qi, Zhou, & Liu, 2013) (Petrou, et al., 2013) (Singh, et al., 2013) to identify such conditions/factors. Despite its potential however, the current use of BM-MSCs to generate clinically relevant auricular tissue constructs is currently rather limited.

2.2.1.2.2 Adipose Derived Mesenchymal Stem Cells

Less studied than bone BM-MSC, adipose tissue derived mesenchymal stem cells seem to harbour mesenchymal stem cells that are capable of undergoing chondrogenesis. A study which compared such cells and their application to cartilage tissue engineering, obtained similar results as groups that had worked with BM-MSC, in that cartilage derived cells yield constructs with more developed constructs (Baptista, et al., 2013). The issue as stated by the authors is that “adipose tissue derived cells revealed an incomplete differentiation into chondrocytes, compared to cartilage derived cells. Similar to BM-MSC, the reason for this could be the lack of a defined path of differentiation for such multipotent cell populations; whereas cartilage derived cells maintain an intrinsic memory of that developmental programming (Estes, Diekman, Gimble, & Guilak, 2010).

2.2.2 Culture Method

2.2.2.1 Pellet Culture

Three-dimensional (3-D) cultures have been shown to promote chondrogenesis (Gadjanski, Spiller, & Vunjak-Novakovic, 2012). The most common and robust method of evaluating the chondrogenic potential of a cell population is to form cell pellets via centrifugation, which provides the cells with the dense, 3-D organization that is known to promote chondrogenesis. Although this method has been shown to generate cartilage, its spherical shape dramatically limits its clinical use. Its easy use has made a primary method of choice for researchers who wish to assess chondrogenic potential of different cell populations, as well as the effects of different chondrogenic factors/medium.

2.2.2.2 Micromass Culture

Micromass cultures are created by suspending a large number of cells in a low amount of volume, and plating in a droplet, which creates a dense multilayer of cells. This dense multilayer mimics early embryogenesis in which mesenchymal condensation occurs at the initiation of chondrogenesis. Thus it is a method that is developed via insights into skeletal development (Mello & Tuan, 1999). As a result, it is a method that is very commonly used in MSC

differentiation experiments. Because it is carried out in a plate as a flat multilayer, its histochemical analysis (usually alcian blue) can also be carried out on the plate, not requiring tissue embedding, sectioning, and slide processing as needed for pellet culture (Liao, et al., 2014). Studies comparing the two methods have shown that micromass cultures are stronger chondrogenic inducers than the pellet system, as micromass constructs show higher production of collagen type II and aggrecan, and lower levels of collagen type I and collagen type X (Zhang, et al., 2010). Alongside such advantages, the potential of micromass cultures for cartilage tissue engineering lies in the fact that it permits shape specific chondrogenesis. Shape specificity of micromass culture arises from the shape of the droplet as well as the shape of the surface of the plate, which can both be easily manipulated via 3-D printing mold. Interestingly, if these micromass cultures are able to undergo full chondrogenesis and produce mechanically stable constructs, then this would be an enormous step forward for the field, as it would provide a means of creating shape specific cartilage constructs without the use of scaffolds. Interestingly, this potential was highlighted in a review by (Handschel, et al., 2007). The authors highlighted the potential of micromass culture technology due to its potential in generating scaffold-free cartilage constructs, However, to our knowledge, the potential of the micromass system has not yet been used to generate auricular cartilage constructs.

2.2.2.3 Scaffold Seeded Constructs

Currently, the most common method for creating shape specific auricular cartilage constructs is through the use of porous scaffolds. Commonly, expanded chondrocytes are seeded into a porous biocompatible/biodegradable scaffold and subcutaneously engrafted in a nude (immunocompromised) animal for 3-6 months in order to generate cartilaginous constructs (Enjo, Terada, Uehara, Itani, & Isogai, 2013) (Sterodimas & de Faria, 2013). This is now considered a popular method for complex tissue regeneration, due to its simplicity of creating complex 3-D structures, such as those of the external ear.

To generate patient specific auricular shaped porous scaffolds, the patients normal ear is 3-D scanned (using various 3-D scanning methods such as CT scans) and displayed in a computer software program which then generates a mirrored image of the normal ear followed by its negative mold. This process is often referred to as Computer Aided Design (CAD). This negative

mold is then 3-D printed through a Computer Aided Manufacturing (CAM) process. Often coupled together, the two are called CAD/CAM as outlined by (Liu Y, 2010). Non-woven fibers of interest (commonly used fibers include: polyglycolic acid, PGA, polylactic acid, PLA, or poly (lactide-co-glycolide), PLGA) are then compressed in the negative mold, generating anatomically specific porous scaffolds (Janjanin, Li, Morgan, Shanti, & Tuan, 2008).

Ideally, construct chondrogenesis and maturation would occur at the same rate as scaffold degradation, resulting in retention of the original scaffold shape/structure. Identifying scaffolds that provide all biocompatibility features necessary for their use in auricular tissue engineering as well as exhibiting the exact degradation time is proving to be a challenging task. For example: PLLA scaffolds have a half-life of roughly 12 months, indicating that 50% of the polymer will still be present after a year of construct implantation (Yamaoka, et al., 2010). Longer half-life of scaffolds could result in impaired tissue maturation, as well as undesired host responses (Yamaoka, et al., 2010), and therefore scaffolds with shorter half-lives need to be developed. However, degradation times must not be greater than construct maturation, as this would jeopardize construct shape retention. Another important component of any synthetic polymer for use in tissue engineering is the degradation by products. For example it is in fact known that PLA has very acidic degradation products, which could harm the construct as well as surrounding tissue (von Burkersroda, Schedl, & Göpferich, 2002). Therefore, although they help scientists generate cartilage constructs easily, their use in patients is not ideal.

2.2.2.4 Hydrogel Based Constructs

Another common method of providing 3-D structural information to chondrocytes is through the use of hydrogels. In this method, cells are suspended in biocompatible hydrogels, which are then injected into a negative mold created as described above in section 2.2.3.5. Similar to synthetic scaffolds hydrogel-cellular constructs are often implanted subcutaneously into immunodeficient animals, and allowed to mature for 1-6 months (Reiffel, et al., 2013). Some hydrogels include Collagen type I, alginate, and atellocollagen (Reiffel, et al., 2013; Hinek, Kawiak, Czarnowska, & Barcew, 1984; Choi, et al., 2008). These types of gels can ultimately have more acceptable clinical outcomes compared to synthetic polymer scaffolds due to the current successful use of

non-autologous biopolymers for clinical purposes (Reiffel, et al., 2013), however, obtaining such high quality hydrogels for use in patients is not ideal.

A major drawback with using synthetic polymers and non-autologous biopolymers to create shape specific auricular constructs via subcutaneous implantation into nude animals is that the immunogenicity of such carriers cannot be fairly evaluated. This is important because immunocompetence of in the host can affect polymer degradation, vascularization, and long term construct stability (Reiffel, et al., 2013). Therefore, to reduce such complications, the ideal construct generation method would avoid the incorporation of any non-autologous components, and would be use only the patient's own cells for safer and more directly applicable clinical relevance.

2.2.3 Mediums and Factors

To date, numerous studies have identified various factors that induce chondrogenesis (Sterodimas A. , de Faria, Correa, & Pitanguy, 2009). Although there currently seems to be a general consensus of what a chondrogenic medium consists of, there are still many studies which aim to enhance chondrogenic stimuli and conditions.

The first parameter that needs to be investigated is the basal medium. Because our study focuses on using paediatric auricular chondrocytes for tissue regeneration purposes, it was crucial that we first identified the appropriate culture medium for these primary chondrocytes. A study carried out by Ruszymah BH (2007) showed that DMEM/F12 medium supplemented with 10% FBS (DF10) showed significantly higher growth rate relative to DMEM and F12 alone , each supplemented with 10% FBS (D10 and F10 respectively). The authors also investigated the expression of the chondrogenic COLII/COLI ratio and aggrecan in *in vitro* monolayer cultures, and found that cells in F10 expressed statistically significant higher levels of these factors during each passage compared to DF10 and D10 alone. It is important to note that the expression of these genes in DF10 mixture was also statistically significant higher than in D10 alone. Interestingly, DF10 showed the highest levels of elastin gene expression compared to the other two media, identified via RT-qPCR reactions. The authors concluded that DF10 mixture was the most advantageous medium to use for human paediatric auricular chondrocyte expansion *in vitro*.

Additionally to DMEM/F12 supplemented with 10% FBS, further factors are being identified which enhance proliferation and reduce chondrocyte dedifferentiation. For example, a study (Liu, et al., 2014) has shown that the addition of (1%) Insulin-Transferrin-Selenium mixture to DF10 will enhance pig auricular chondrocyte proliferation rate as well as construct formation by increasing construct size, wet weight, and ECM components. Another Study (Xu, et al., 2011) has shown that the supplementation of recombinant human midkine, previously identified to affect neuron outgrowth, tumour progression, and bone fracture healing, stimulates proliferation of rat primary auricular chondrocytes *in vitro*, and also helps chondrocytes maintain their chondrocyte specific expression of matrix protein throughout *in vitro* monolayer culture expansion. Therefore, there are ongoing investigations which aim to increase the slow proliferation of chondrocytes, including tackling the challenge of *in vitro* dedifferentiation of chondrocytes.

Post *in vitro* monolayer expansion, cell culture conditions need to be changed in order to redifferentiate dedifferentiated chondrocytes. As described in earlier chapters, high cell density cultures such as pellet cultures, and micromass cultures are commonly employed, however, additional chondrogenic stimuli is often required for tissue formation besides high cell density cultures.

A large effort in the field lies in identifying chondrogenic factors which enhance ECM deposition, ultimately yielding more mechanically stable constructs. Studies have shown that *in vitro* ECM production is significantly enhanced during differentiation under serum-free conditions (Bilgen, Orsini, Aaron, & Ciombor, 2007) rather than in serum containing conditions. Such studies have commonly used DMEM as the basal medium. Supplementing DMEM with additional factors has been shown to aid *in vitro* chondrogenesis. Perhaps the most important and potent chondrogenic factor that is often supplemented to aid redifferentiation is Transforming Growth Factor ($\beta 1$ and $\beta 3$) (Wang, Kim, Blasioli, Kim, & Kaplan, 2005). Dexamethasone has been shown to enhance chondrogenesis of MSC, shown by an increase collagen type II and GAG content (Stewart, Byron, Pondenis, & Stewart, 2008; Wang, Kim, Blasioli, Kim, & Kaplan, 2005). Salts of ascorbic acid have been known to upregulate collagen deposition for a long time (Murad, et al., 1981). ITS premix supplementation is often required for serum free medium

(Kisiday, Kurz, DiMicco, & Grodzinsky, 2005). Finally 1X antibiotic/antimycotic is commonly added to the medium to prevent bacterial and fungal contaminations of constructs.

Interestingly, multiple studies have revealed that additional to the composition of the chondrogenic differentiation medium, the temporal supplementation of such factors is crucial for chondrogenesis as well. Multiple groups have shown that the transient supplementation of TGF- β enhances chondrogenesis and tissue construct maturation (Ng, O'Connor, Kugler, Cook, & Ateshian, 2011; Byers, Mauck, Chiang, & Tuan, 2008).

Although multiple additional factors have also been identified to aid chondrogenesis, however none are as widely used as TGF- β . Perhaps the most important of these for auricular tissue regeneration is Insulin like Growth Factor -1 (IGF-1). IGF-1 is a growth factor that has been identified as a key stimulus for elastogenesis in elastogenic cells such as lung fibroblasts and cardiac smooth muscle cells (Mariani, Sandefur, & Pierce, 1997). Studies analyzing the chondrogenic potential of IGF-1 have reported that IGF-1 enhances ECM deposition (Ng, O'Connor, Kugler, Cook, & Ateshian, 2011).

2.2.4 Bioreactor cultivation of tissue engineered auricular cartilage

In order to generate 3D tissue that is biologically significant in thickness, bioreactors are commonly employed. Bioreactors are able to achieve this by increasing the rate of toxic waste removal and nutrient available by providing constant flow of medium. Giardini-Rosa et al. (2014) investigated the effects of a continuous flow bioreactor system on primary rabbit auricular chondrocytes, with promising results. Others have used the Rotary Cell Culture System (RCCS) which allows the suspension of a solid mass within a rotating vessel while oxygenating the medium through a silicone gas transfer membrane (Mellor, Baker, Brown, Catlin, & Oxford, 2014). The RCCS is particularly useful as it allows for the free suspension of 3D complex constructs, ideal for auricular tissue generation, however, its effects on chondrogenesis seem to vary with culture conditions.

Previous studies investigating the roll of micro gravity culture conditions on chondrogenesis have produced inconsistent results. Groups comparing pellet cultures of human mesenchymal stem cells grown in bioreactor vs. static cultures have shown that the RCCS in fact negatively

impacts chondrogenesis (Mayer-Wagner, et al., 2014), while others have reported that the RCCS does not enhance the re-differentiation of rat articular chondrocyte/alginate-agarose hydrogel constructs (Barlic & Kregar-Velikonja, 2008). In contrast, other studies report that microgravity stimulation of bone marrow mesenchymal stem cells seeded PGA scaffolds by RCCS enhances toluidine blue staining (corresponding to increased GAGs), as well as significantly increases collagen type II mRNA levels (Wu, Li, Lou, & Chen, 2013). Other studies have also shown similar results, where microgravity stimulation of adipose derived mesenchymal stem cells promotes chondrogenesis (Yu, et al., 2011).

2.2.5 Human Studies

Due to the difficulty of obtaining human cartilage tissue samples, human auricular cartilage regeneration studies have been few. This is as expected due to the scarcity and difficulty of obtaining human tissue, especially of paediatric patients.

Over the last decade the tissue engineering approaches that were developed inside the laboratory have begun to find their way into the clinic, mainly by Dr. Yanaga and his group in Japan. This group isolated chondrocytes from auricular cartilage, expanded them *in vitro* using auto-serum from each specific patient and a multilayer technique which creates more dense cultures, known to promote chondrogenesis, and subsequent subcutaneous implantation. It is important to note that for these clinical practices, Yanaga et.al. did not rely on the use of any sort of scaffold or carrier gel to shape the tissue. Dr. Yanaga and his group still relied on surgical expertise to shape the desired auricular framework. His methods showed great promise for the translation of the current advancements in the field of auricular tissue engineering. When these tissue constructs were analyzed histologically and immunohistochemically, it was revealed that they exhibited a very similar architecture and ECM composition as native cartilage, with the abundant presence of GAGs, Collagen type II, and Elastin (Yanaga, Imai, Fujimoto, & Yanaga, 2009; Yanaga, Imai, Koga, & Yanaga, 2012).

2.3 Rationale

Current treatments available to patient suffering from microtia is limited, and mainly requires large autologous cartilage grafts which leave donor site morbidities, while providing suboptimal

results. Tissue engineering holds a promise to be able to provide surgeons with more accurate cartilage grafts using smaller tissue harvests. Significant advances have been made utilizing animal cell sources to generate shape specific constructs; however, translational studies using normal human paediatric auricular tissue are few. In this thesis, my goal was to solely use chondrocytes from human paediatric auricular cartilage tissue to generate mechanically stable, scaffold-free auricular constructs *in vitro*, which display the full spectrum of auricular cartilage tissue (cartilaginous component wrapped by surrounding perichondrium). This has three main advantages 1) using human paediatric samples ensures translational findings 2) scaffold-free tissue engineering minimizes the possibility of construct rejection by host, and 3) due to *in vitro* development of paediatric auricular tissue displaying the full spectrum of auricular tissue, eliminated the need of any prior subcutaneous implantations. Due to these advantages, *in vitro* scaffold-free tissue generation provide the ideal clinical application of cartilage tissue engineering for patients in need of auricular and nasal reconstruction surgeries demand additional tissue.

2.4 Hypothesis

Human paediatric chondrocytes can be used to generate scaffold-free cartilaginous constructs *in vitro* using multilayer culture systems

2.5 Objective/Aims

The objective of my study was to evaluate the potential of human paediatric auricular chondrocytes to form scaffold-free cartilaginous constructs *in vitro*.

To test this hypothesis, the specific aims are:

- Aim 1) Establish human paediatric native tissue endpoints. Analyzing native tissue for histological, immunohistochemical, mechanical and biochemical properties would provide ideal goals for tissue construct generation.

- Aim 2) Establish patient specific chondrocyte cell sourcing, and evaluate the *in vitro* chondrogenic potential of these patient specific chondrocytes in scaffold-free multilayer culture systems.
- Aim 3) Evaluate the effects of Rotary Cell Culture System on multilayered chondrocyte constructs.

Chapter 3

Generating Scaffold-Free Cartilage Constructs resembling native Paediatric Auricular Cartilage

3.1 Introduction

Current treatments of auricular deformities rely on the use of autologous costal cartilage harvests, or magnetic/clip on prosthetics. Autologous cartilage grafts harbour great donor site morbidity and inaccurate patient specific auricular shapes due to the difficulty of the surgical procedure, whereas prosthetics have high implant failure rates, are susceptible to infections, and lack human like texture and feel. In contrast to these suboptimal treatment options, auricular tissue engineering offers a possible solution by allowing the generation of accurate patient specific auricular shaped cartilage constructs with minimal tissue harvests and thus surgical complications. However, despite such great potential, there are many technical challenges which remain to be overcome before the field is able to offer consistent and reliable treatment ready auricular tissue to patients in need.

Developing auricular cartilage with great shape specificity and accuracy has proven to be difficult *in vitro*. Currently, the best systems for recapitulating the native auricular shape has been achieved by cell seeding polymer scaffolds and subcutaneous implantation in host for tissue maturation (Sterodimas A. , de Faria, Correa, & Pitanguy, 2009). Although some have reported auricular cartilage generated with shape precision (Zhang, et al., 2014), shape retention is commonly poor due to scaffold degradation as well as possible host inflammatory responses leading to construct rejection (Sung, Meredith, Johnson, & Galis, 2004; Gilpin, Weidenbecher, & Dennis, 2010). Due to this, the search for the ideal method to generate stable constructs resembling native tissue continues.

In order for a functional auricular construct to successfully integrate into the host, it required a functional perichondrium (Giardini-Rosa, et al., 2014). Without a perichondrium, integration could be significantly delayed, leading to construct starvation and ultimately failure. Typically, to overcome this challenge, constructs are implanted subcutaneously prior to the actual auricular

reconstruction procedure. In this chapter, I will describe our novel method of generating auricular cartilage constructs from human paediatric auricular tissue without the use of any scaffold, displaying the presence full presence of perichondrium similar to that of native tissue. Using micromass culture technology coupled with prolonged induction periods, we were able to generate plates of cartilaginous constructs which histologically, immunohistochemically, mechanically, and biochemically resembled native paediatric auricular cartilage.

In order to successfully bioengineer a construct that accurately resembles and can ultimately function like a native ear, we must first study and analyze in detail the design and architecture of the organ. We propose that preliminary studies be carried out which will focus on determining the overall cellular structure of the ear. This is crucial; because once the ex-vivo culture is underway, the project needs to be aimed towards accomplishing a structure that histologically and mechanically resembles a native ear. This approach will serve to fine tune all subsequent aspects of the tissue engineering portion of the project, and will help guide strategic experimental designs.

3.1.1 Materials and Methods

3.1.1.1 Whole auricular tissue sectioning

Due to the inconsistency of surgical samples received from the operating room, it was impossible to systematically analyze all the different portions of the pinna and develop auricular endpoints which we could confidently state to be representatives of all the different areas of the pinna, as well as the total population with respect to age, and sex. Therefore, in order to validate endpoints, the REB was modified to grant permission to obtain cadaveric tissue from the Department of Anatomy, University of Toronto, and transfer them to the Hospital of Sick Children. Upon REB amendment, a total of 6 cadaveric ears were obtained. These cadavers were 3 female and 3 male. All cadaveric ears were available fully embalmed; details pertaining to cadaveric pinna are summarized in **Table 3-1**.

In order to evaluate and compare different portions of the pinna, auricular segments were segregated based on early auricular development (Hillocks of His), highlighted in section 1.2 and **Figure 1-1**. The common consensus for the adult structures and their respective Hillocks of His is outlined in **Figure 3-1**.

3.1.1.2 Auricular cartilage thickness measurements

Selected areas representing each Hillock of His were excised out and cartilage measurements were obtained using a digital caliper. Due to varying thicknesses even within each segment the, 4 different measurements were made for each piece and the average was used as a representative. These results are summarized in **Table 3-3**.

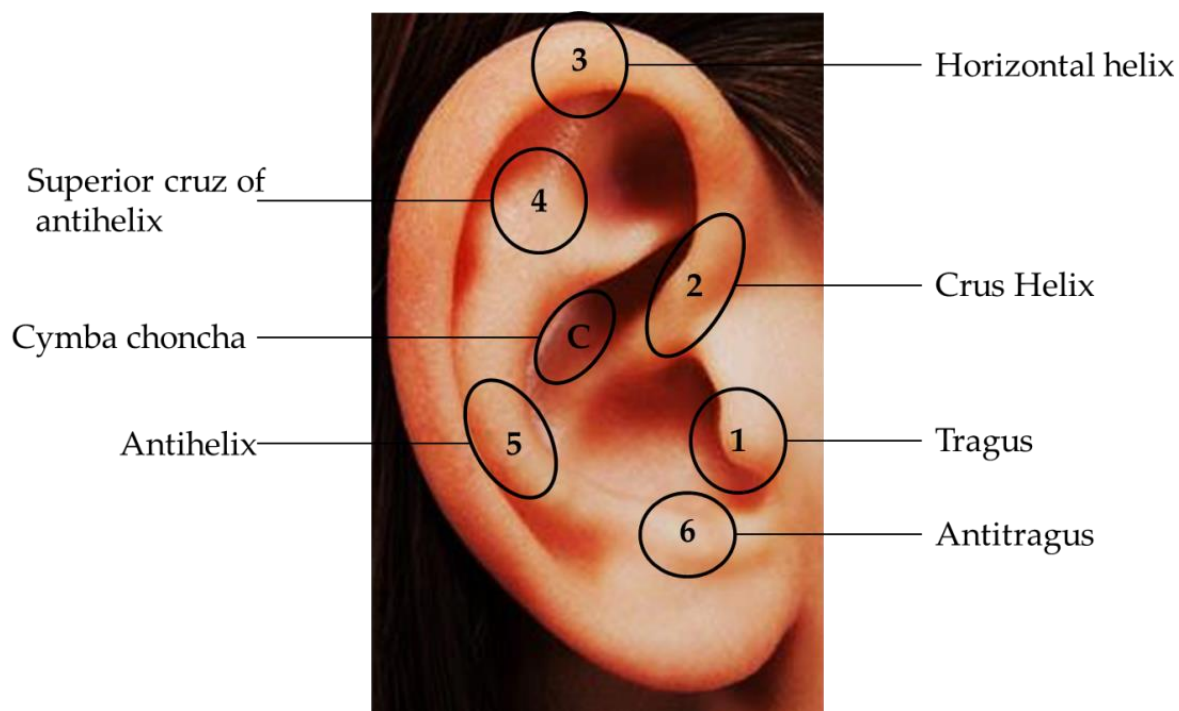
3.1.1.3 Histology

Cartilage tissue samples to be processed for histology and immunohistochemistry were fixed in 10% formalin for 48 hours, embedded in paraffin and sectioned into 5 μ m sections and placed onto treated microscope slides. Sections were deparaffinized by three 10 minute xylene washes, hydrated through a graded series of ethanol (100%, 70%, 50%, and 10%), until brought into ddH₂O. Slides were then stained with hematoxylin and Eosin (H&E, a general tissue stain,

safranin O (sulphated proteoglycan stain), and either elastin trichrome (elastic fiber stain) or movat's pentachrome (elastic fiber stain) (Reiffel, et al., 2013; Hinek, Kim, Wang, Wang, & Mitts, 2014).

Table 3-1. Cadaver tissue information

Specimen (Code)	Sex	Age-at-death
7022	F	51
6540	F	86
7028	F	86
7028	M	74
7056	M	80
7012	M	84

**Figure 3-1.** Cadaver auricular sectioning

Cadaver pinna were sectioned based on the assignment of different auricular features to their embryological origin (Hillocks of His) as well as the concha.

3.1.1.4 Immunohistochemistry

COL I: After deparaffinization and hydration, sections were subjected to 10 mM sodium citrate pH 6.0 antigen retrieval at 100°C for 15 minutes. Endogenous peroxidase activity was quenched with 3% hydrogen peroxidase in H₂O treatment for 15 minutes. Tissue was then digested for 15 minutes with preheated (37°C) Pepsin digest mix (Life Technologies). Non-specific binding was quenched via a blocking step with 5% horse serum blocking step for 1 hour. Sections were then incubated with mouse monoclonal anti-collagen type I antibody (Abcam: ab6308) at 1:400 and 4°C overnight, followed by the Vectastain anti-mouse ABC kit conjugated with HRP. Finally sections were incubated with the HRP substrate diaminobenzidine (DAB; Vectastain) for 6 minutes. After DAB, sections were counterstained with Mayers Hematoxylin for 40 seconds.

COL II: COL I immunohistochemistry protocol was used collagen type II staining, using anti-mouse collagen type II antibody (Millipore: MAB8887) at 1:200 dilution.

All cadaveric histological and immunohistochemical processing was performed following the same protocol as native paediatric auricular tissue, described in 3.1.1.

3.1.1.5 Human Paediatric Primary Chondrocyte Isolation, Culture, and Expansion

In order to obtain human paediatric samples through the Hospital for Sick Children Otolaryngology surgical team, a Research Ethics Board (REB) application was submitted in September 2013, and approved in December 2013 (REB#1000039790). This REB application contained research protocols, consent forms, and an internal REB application form. Importantly, this REB allowed me to obtain otherwise discarded surgical remnants from any type of ear, nose, and throat surgical procedures performed by the Department of Otolaryngology.

From December 2013 to April 2015, a total of 18 usable auricular samples were obtained, for which the demographic is shown in **Table 3-2**. Samples Cartilage samples that were remnants of cholesteatoma surgeries were considered to be normal, since these children exhibit normal external ears, as well as normal metabolic functions. Samples varied not only from patient to

patient in terms of gender and age, but also by anatomical resection sites. Thus the cohort permitted trialing the heterogeneity represented in the paediatric population.

Cartilage tissues were viewed under a dissection microscope and soft connective tissue and fat tissue were stripped away using adson forceps and microdissection forceps, and a sharp scalpel. Tissues were minced into 1 x 1 mm pieces with a sharp scalpel blade, and washed three times with PBS containing 1X antibiotic/antimycotic. Minced tissue was then placed into DMEM high glucose with 1X antibiotic/antimycotic containing 0.25% collagenase type II (Worthington Biochemical Corporation) for 16 hours overnight at 37°C and 5% CO₂. Digested tissue was passed through a 100-µm nylon mesh filter (Falcon, Franklin Lakes, NJ, USA), centrifuged, and washed with DF10, and centrifuged again to obtain a small cell pellet free of digestive enzymes and undigested tissue. Harvested cells were counted and % viability was calculated using a hemocytometer and trypan blue dye exclusion. Primary human auricular chondrocytes (passage 0, P0) were seeded at a density of 5×10^3 cells/cm² into tissue culture treated six-well plates and cultured in DF10 medium. Medium was changed once every two days until cultures became 80% confluent. Cells were passed by first washing with PBS (not containing Mg²⁺ Ca²⁺) for 1 minute, and then incubated for 5 minutes with 0.025% trypsin containing 0.02% EDTA (Wisent Bio Products) to promote dissociation of the cells. Plates were then scraped with a sterile cell scraper to free any remaining loosely bound cells, maximizing chondrocyte harvest. Cells were passaged at 1:3 ratio generating passage 1 (P1) cultures. Culture conditions as well as cell passaging was performed the same way for all subsequent passages.

Table 3-2. Human paediatric auricular tissue sample demographic.

Sample #	Age	Gender	Tissue Location
1	14	F	Tragus
2	10	M	Tragus
3	15	M	Concha
4	5	F	Tragus
5	13	F	Tragus
6	7	M	Concha
7	7	M	Tragus
8	5	M	Concha
9	14	M	Tragus
10	13	M	Concha
11	11	M	Concha
12	9	F	Concha
13	14	M	Concha
14	8	M	Tragus
15	9	M	Concha
16	10	F	Tragus
17	13	M	Tragus
18	7	M	Concha

3.1.1.6 Aggregation Assay

To assess the behaviour of passage 3 (P3) expanded auricular chondrocytes to chondrogenic stimuli in monolayers, P3 expanded chondrocytes were exposed to serum free chondrogenic medium containing DMEM/F12 basal medium supplemented with 1x ITS mixture (Life Technologies), 10ng/ml TGF- β 3 (R&D Systems), 0.1 μ M dexamethasone, and 50mg/ml ascorbate (both from Sigma-Aldrich). Results are shown below in **Figure 3-8**.

3.1.1.7 Micromass culture generation

Passage 3 cells were harvested at 80% confluency by trypsinization as described in section 3.1.1.5. Harvested cell pellets were suspended in DF10 at a density of 5×10^4 cells/ μ l, and 50 μ l droplets were gently pipetted into the center of a twelve-well plate. Micromass cultures were left undisturbed inside the laminar flow hood for 5 minutes to allow cells to settle at the bottom of the droplet. The plate was then carefully placed inside the incubator at 37 °C and 5% CO₂, and cells were given 2.5 hours to attach. The micromass culture generation is illustrated in **Figure 3-9**. Cartoon depiction of micromass culture formation Upon cellular attachment, 2 ml of DF10 was carefully pipetted into the well through the sides of the well as to not disturb the micromass culture. The plate was then carefully transferred to the incubator and culture was left for 24 hours to complete the process of cellular attachment. The medium of each well was then replaced every two days, twice. After 4 days, this medium was replaced with the chondrogenic differentiation medium described in section 3.1.1.6. With every media change, monolayer cells surrounding the micromass cultures were scraped off and aspirated using the tip of a 1 ml pipette tip. After 4 weeks of chondrogenic induction, the micromass was carefully lifted off the plate using a flat weighing spoon, if not already lifted by itself during the chondrogenic induction. After 4 weeks of chondrogenic induction, factor containing medium was replaced with DMEM/F12 containing 1x ITS premix, and 1x antibiotic/antimycotic. Upon lifting of the micromass culture from the plate, continuous attempts at physically lifting constructs off of the plate were made, and the mechanical stability of constructs were qualitatively analyzed, as shown in **Figure 3-10**.

3.1.1.8 Mechanical testing and properties

Micromass engineered constructs show exhibit shape retention and mechanical stability, as they could be readily lifted from the plate with forceps, while retaining their shape, as shown in **Figure 3-10 B**. To compare the mechanical stability of the engineered tissue to that of native tissue, quantitative parameters which measure different aspects mechanical properties of both native and engineered tissue were analyzed and compared, in the lab of Dr. Stephen Waldman.

The thickness of the tissue constructs was determined using a digital caliper over three random locations of the constructs. Mechanical testing of native auricular cartilage and engineered cartilage constructs was performed using a Mach-1 Micromechanical Testing system (Biomomentum, Laval, QC, Canada) equipped with a 1 kg load cell. Tissue mechanical properties (elastic modulus and Poisson's ratio at 37°C in DMEM/Ham's F12 media) were determined using a double compressive indentation method using two plane-ended indenters (4 mm diameter). Compressive indentations were conducted at a ramp rate of 10% strain/s to a maximum of 10% strain. The resulting force-deformation response (collected a frequency of 10 Hz) were then used to determine the elastic modulus and Poisson's of engineered auricular cartilage ratio of samples using custom-designed code based on the theoretical model of cartilage indentation (Giardini-Rosa, et al., 2014).

3.1.1.9 Biochemical properties of native and engineered tissue

Biochemical quantification of matrix components in the engineered constructs was performed in the lab of Dr. Stephen Waldman. Samples were weighed to determine the mass of the entire developed construct. Samples were then digested by papain (bioreactor samples: 40 mg/ml; native tissue samples: 80 mg/ml in 20 mM ammonium acetate, 1 mM EDTA and 2 mM dithiothreitol) for 72 hours at 65°C. Aliquots of the digest were assayed for DNA, proteoglycan and collagen content. DNA content was quantified using Hoechst 33258 dye (Sigma-Aldrich) assay. The proteoglycan content was estimated by quantifying the amount of sulphated glycosaminoglycans using 1,9-dimethylmethylene blue (DMMB) dye binding assay (Sigma-Aldrich). Total collagen content was determined by the determination of hydroxyproline content. Briefly, aliquots of the papain digest were hydrolyzed in 6 N HCl for 18 hours at 110 °C and the hydroxyproline content was determined in the hydrolyzate using chloramine-T/Ehrlich's reagent

assay. Total collagen content was estimated assuming hydroxyproline accounts for 10% of the total collagen mass in cartilage (Giardini-Rosa, et al., 2014).

3.1.1.10 Statistical Analyses

To establish paediatric auricular cartilage tissue endpoints samples from 5 different patients were used. To examine adult auricular tissue, 6 cadaveric auricles were obtained, with 3 female and 3 male. Data for aggregation assay was collected from 8 patients, with 3-6 replicates of each. Samples from 6 were used to construct 1-5 micromass cartilage constructs. Samples from different patients were not mixed at any point in the study. All data were expressed as mean \pm standard error of the mean (SEM). Student's t-test was used to compare data, and p-values less than 0.05 were considered significant.

3.1.2 Results

3.1.2.1 Histological and Immunohistochemical Analysis of Normal Human Auricular Tissue

3.1.2.2 Paediatric

Histological and immunohistochemical results of native paediatric auricular tissues are shown in **Figure 3-1** H & E staining highlights the overall cellular architecture observed in the auricular cartilage. Collagen type I (COL I) immunohistochemistry shows COL I to be minimally expressed in the chondrium of the tissue; however its expression is increased noticeably towards the perichondrium. Collagen type II (COL II) immunohistochemistry shows high levels of COL II in the chondrium only. The expression of COL I and COL II seem to be complementary. Safranin O staining revealed the abundance of sulphated GAGs all throughout the chondrium, and absence in the surrounding tissue. Elastin trichrome staining revealed high concentrations of elastic fibres, depicted by the red arrow in **Figure 3-1**. These fibres are mostly concentrated at the very edge of the lacunae, and scarcely dispersed outwards into the ECM. The expression of elastin, very similar to COL II and GAGs is mainly observed throughout the chondrium, and not in the perichondrium.

3.1.2.3 Adult/Cadaveric

Adult cadaver auricular tissue analysis showed that auricular cartilage ECM components remain intact throughout the course of a lifetime. Tissues showed high levels of sulphated GAGs (**Figure 3-4**), elastic fibers (**Figure 3-5**), and collagen type II (**Figure 3-6**), and low levels of collagen I (**Figure 3-6 B**). These levels of expression are in line with that observed in paediatric auricular cartilage (**Figure 3-2**). Unfortunately, since cadaveric tissue was only available as formalin fixed tissue, biochemical and mechanical evaluations could not be performed. Thus I was not able to quantitatively compare cadaver tissue both internally (comparing different segments of the same pinna) from the same individual, and also comparing the same auricular segments between individuals, and ultimately comparing cadaveric tissue to paediatric tissue) and externally. Due to the state of the tissue, only qualitative comparisons could be performed.

Table 3-3 shows that anatomically distinct portions of the ear exhibit different thicknesses of auricular cartilage. The same auricular structures also show different thicknesses amongst different individuals. This is very important information that tissue engineers need to be aware of, since any method of tissue development would have to take into account this difference and therefore have the means of developing such non-linear tissue. It is important to note that due to the state of cadaver tissue, immunohistochemical staining could not be performed for cadavers 7012, 7028, and 7056.

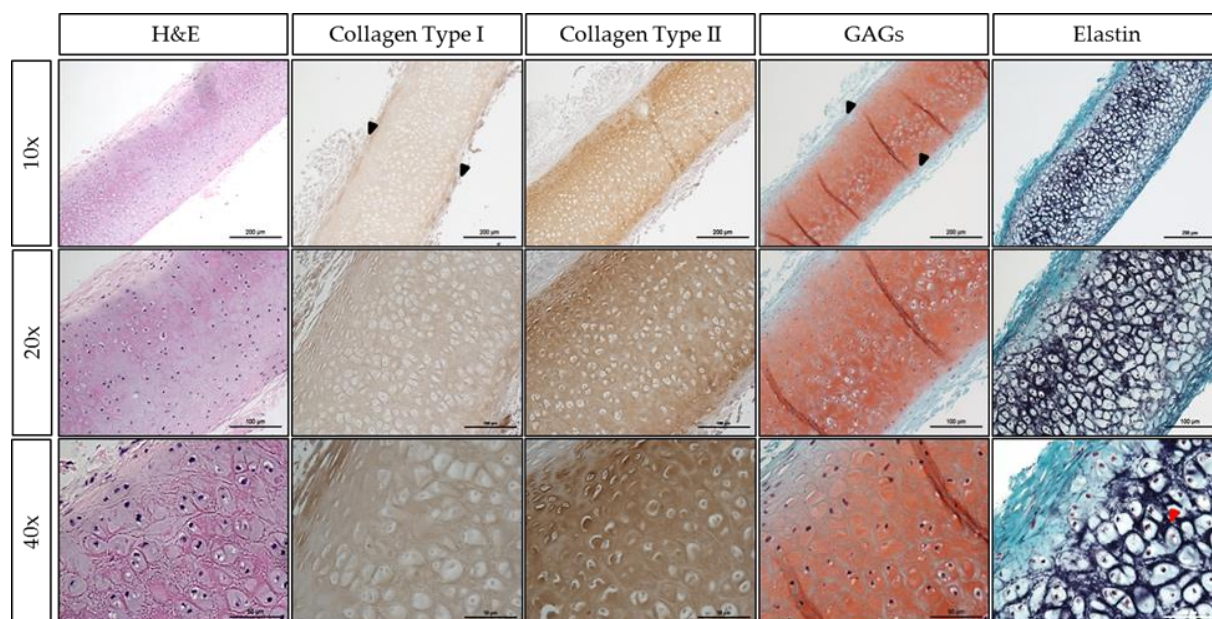


Figure 3-2. Normal paediatric auricular cartilage endpoints.

Representative sample of paediatric auricular cartilage: Tragal cartilage sample from 11 year old Male. Hematoxylin and Eosin stain highlighting overall structure. Immunohistochemistry of Collagen type I. Immunohistochemistry of Collagen type II. Safranin O stain highlighting the presence of sulphated GAGs is orange/red. Elastin trichrome highlighting the presence of elastic fibers is dark blue/black.

Table 3-3. Cadaver auricular cartilage thicknesses (mm) with respect to anatomical location of each structure.

		Cadaver code					
		7056	6928	6540	7028	7012	7022
Auricular Segment	1	1.62	1.65	1.60	1.85	1.75	1.09
	2	1.25	1.07	1.03	1.17	1.43	0.65
	3	1.07	1.12	0.98	1.12	1.28	0.63
	4	1.39	1.26	1.21	1.25	1.24	0.73
	5	1.74	1.36	1.03	1.24	1.65	0.94
	6	1.68	1.34	1.49	1.55	1.66	0.90
	7	1.80	1.08	1.18	1.20	1.45	0.71

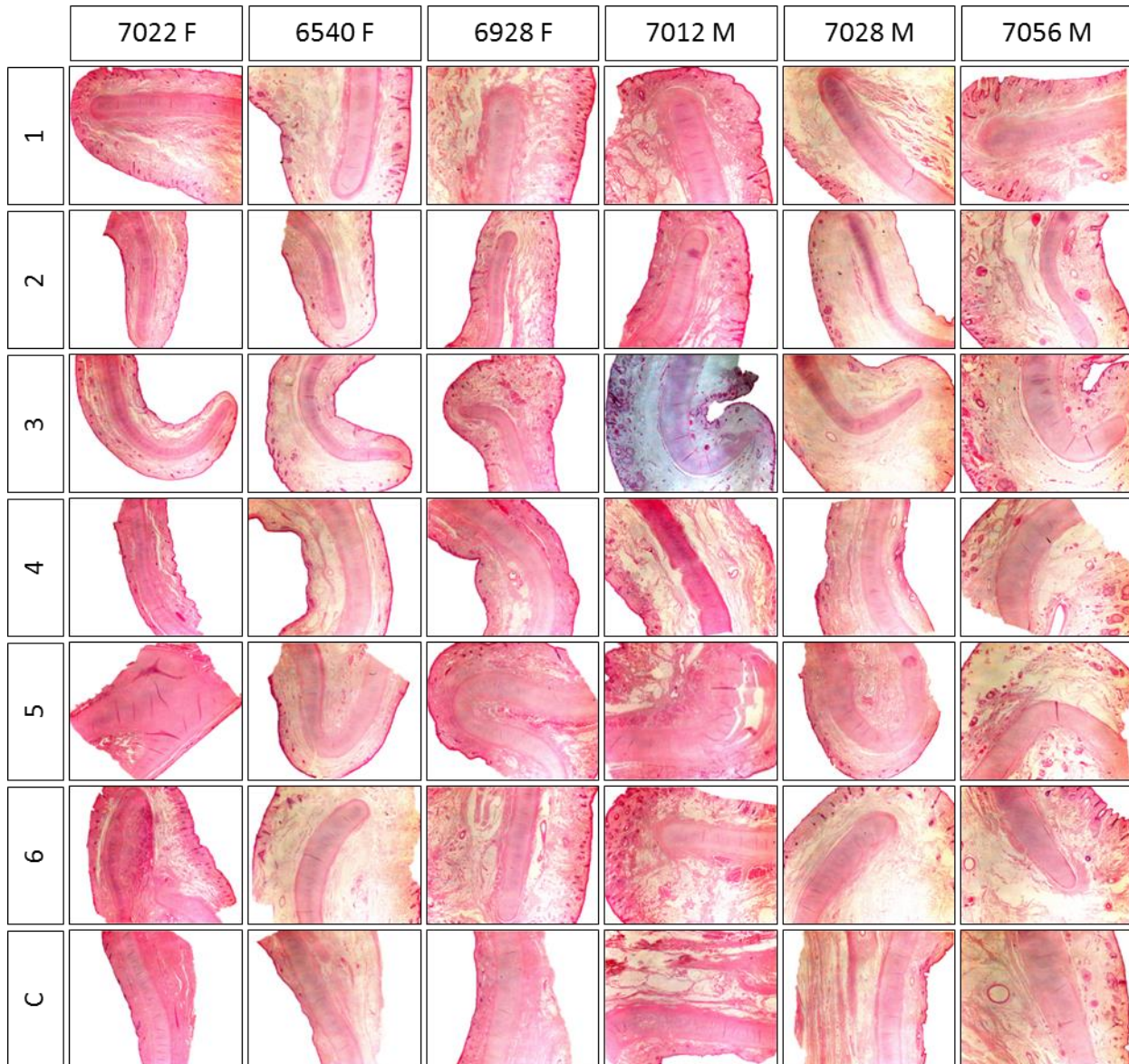


Figure 3-3. Cadaveric auricular cross sections stained with haematoxylin and Eosin stain

Haematoxylin and Eosin (H&E) staining of cadaveric pinna indicating the overall structure of sections corresponding to (1-6) Hillocks of His, and concha. Scale bar: 2 mm.

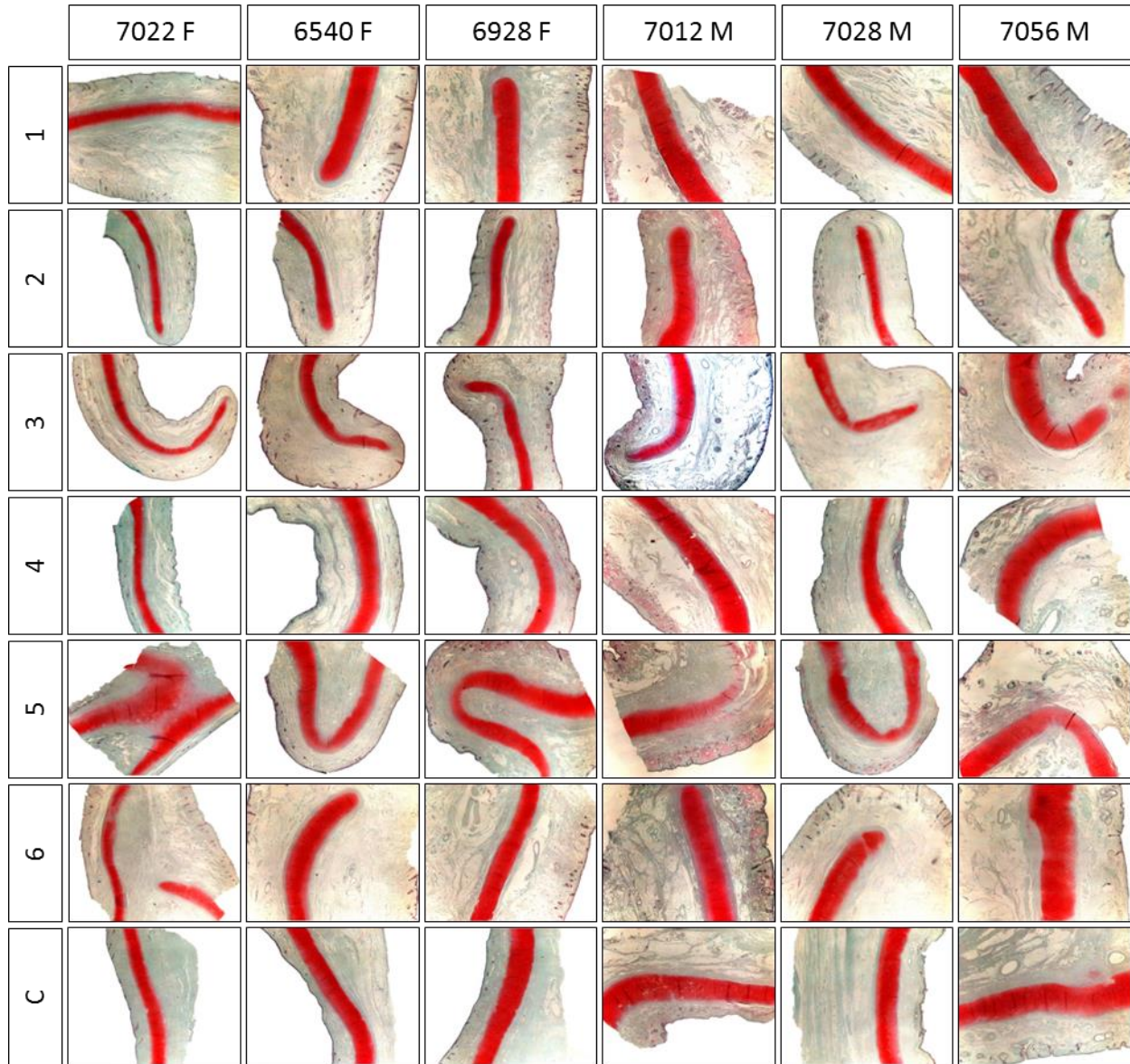


Figure 3-4. Cadaveric auricular cross sections stained with Safranin O stain.

Safranin O staining of cadaveric pinna indicating the abundance of GAGs (orange/red) of sections corresponding to (1-6) Hillocks of His, and concha. Scale bar: 2 mm.

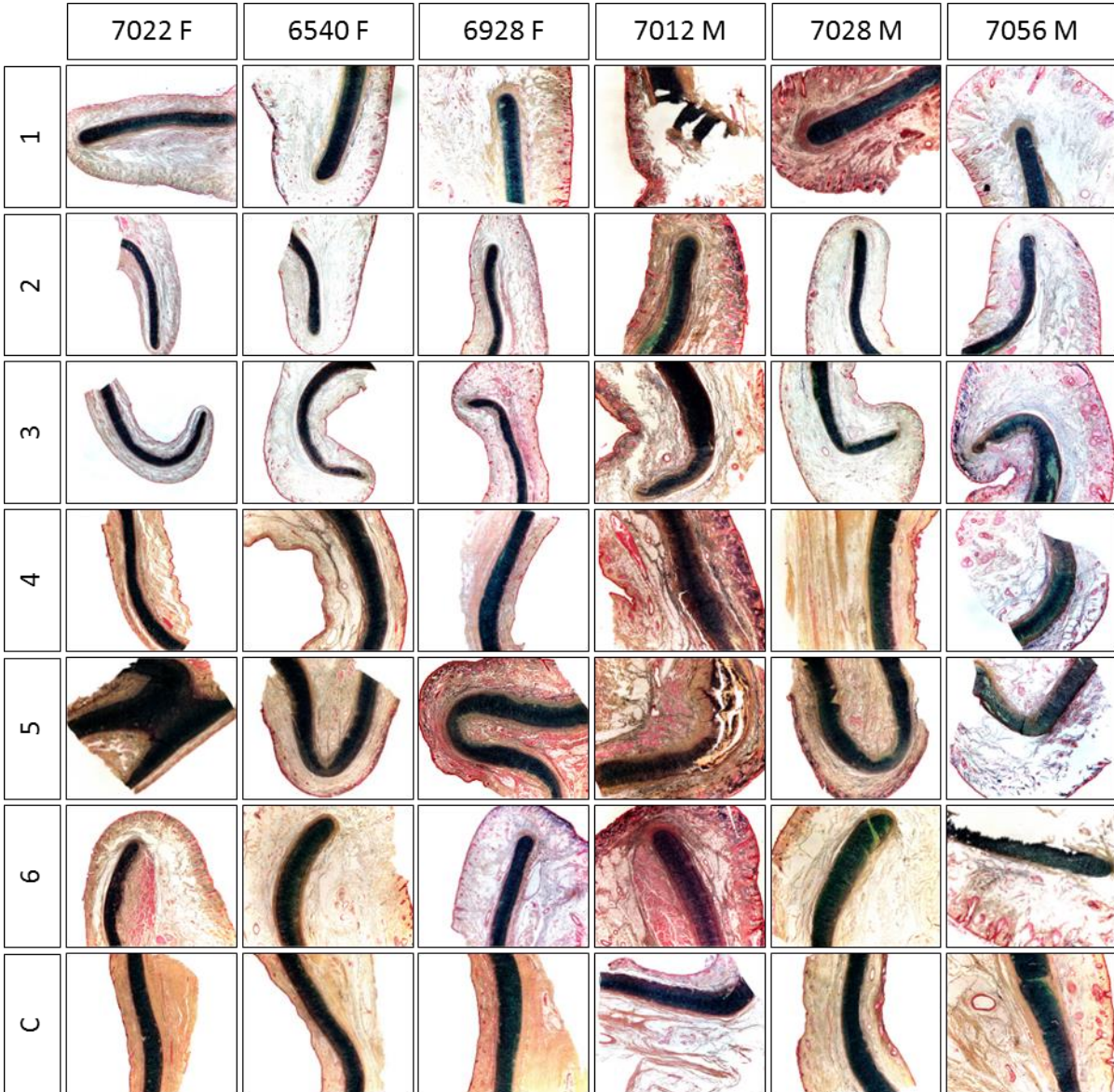


Figure 3-5. Cadaveric auricular cross sections stained with elastin trichrome.

Movat staining of cadaveric pinna indicating the abundance of elastic fibers (black) of sections corresponding to (1-6) Hillocks of His, and concha. Scale bar: 2 mm.

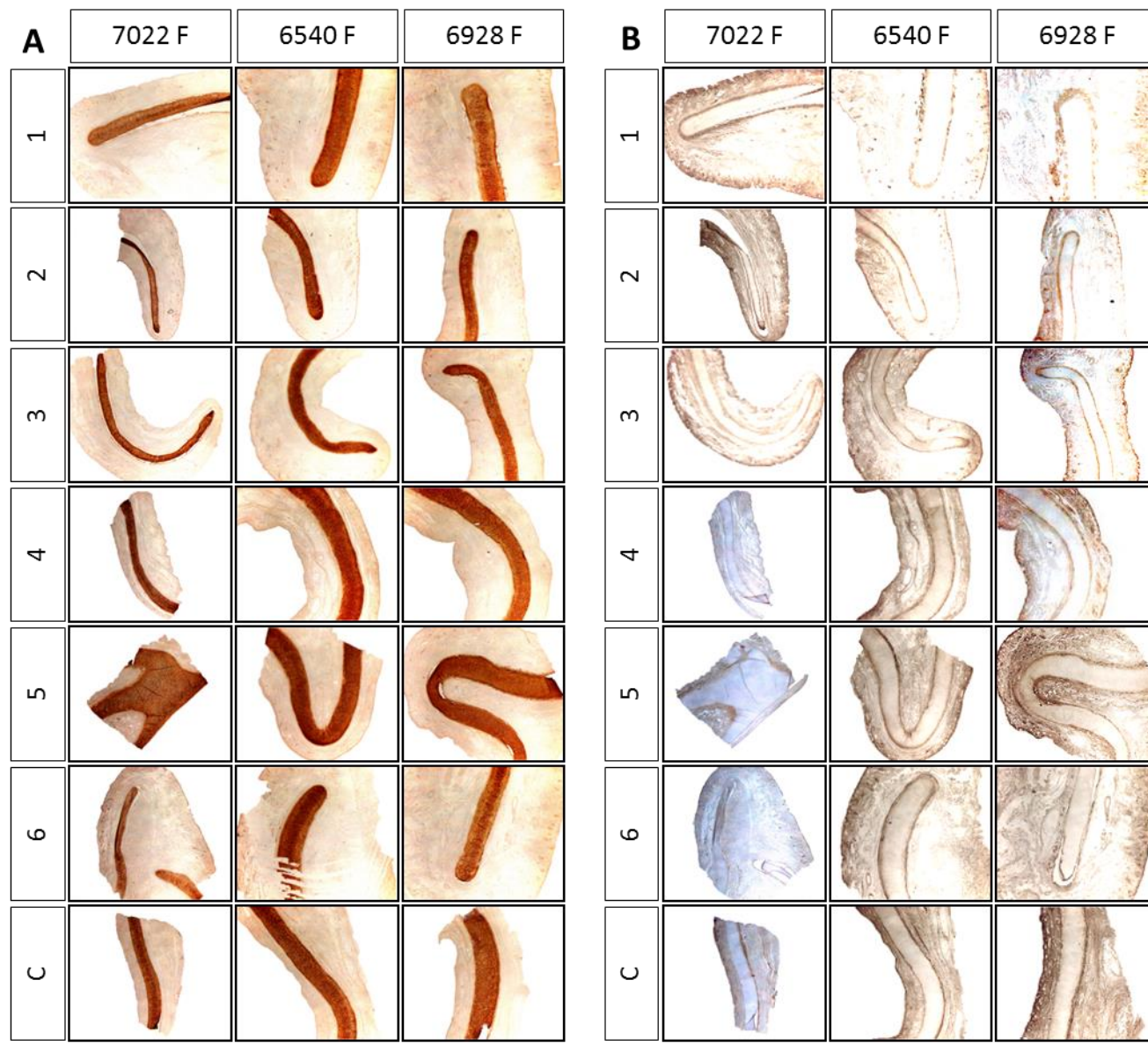


Figure 3-6. Cadaveric auricular cross sections stained for Collagen type II and I.

Immunohistochemical labelling of cadaveric pinna cross-sections highlighting the presence of A) Collagen type II and B) Collagen type I, in sections corresponding to the (1-6) Hillocks of His, and the concha. Scale bar: 2 mm.

3.1.2.4 Monolayer Culture and Aggregation Assay

Primary paediatric auricular chondrocytes showed heterogeneity, displaying the presence of both round chondrocyte-like cells, as well as more spindle shaped fibroblast-like shaped cells, showed in **Figure 3-7**. Expanded paediatric auricular chondrocytes in monolayer exposed to chondrogenic stimuli aggregated overnight **Figure 3-8**.

3.1.2.5 Overall Morphological Appearance

Micromass cultures began to change their appearance into the third week of chondrogenic induction, and become dense and opaque in appearance. After the differentiation period described in 3.2.2.2.1, constructs retained their original circular shape, and could be freely moved. These constructs showed sufficient mechanical stability when lifted with tweezers as shown in **Figure 3-10**. Micromass constructs produced were 0.8-1.0 cm in diameter, and 0.5 mm in thickness.

3.1.2.6 Mechanical Properties of Constructs

Engineered tissue constructs generated with 2×10^6 cells averaged 0.46 ± 0.02 mm in thickness, commonly found in native auricular cartilage, as shown in **Table 3-1**. Poisson's ratio of engineered tissue, which is a measure of the ratio of the lateral compression of the tissue with respect to the longitudinal strain, varied by 34% from native tissue, however with no statistical significance ($P=0.09$). Constructs showed a lower resistance to uniform pressure compared (Bulk modulus) to native tissue by 66% ($P = 0.061$), however this did not prove to be significant. The elastic modulus of the constructs however, did prove to be 51% lower than of native tissue ($p=0.048$). All results are summarized in **Table 3-1**.

3.1.2.7 Histology and Immunohistochemistry

Micromass engineered tissue constructs were subjected to histological, and immunohistochemical analysis. Cartilaginous ECM components analyzed were collagen type I, collagen type II, sulphated glycosaminoglycans, and elastic fibers. These results are shown in **Figure 3-11**. To summarize, micromass constructs showed structural similarities to native

paediatric cartilage, shown in **Figure 3-2**. H&E staining shows rather similar overall morphology of tissue, except native tissue seems to show the presence of thick fibers, whereas micromass cultures do not. By immunohistochemical analysis, micromass constructs appear to contain components of native cartilage, the chondrium and the perichondrium. Micromass constructs exhibited a relatively low level of collagen type I throughout the inner chondrium layer, and high levels of collagen type I throughout the perichondrium, indicated by two black arrows in **Figure 3-11**. Collagen type II levels were high all throughout the cartilage constructs, similar to native tissue. The presence of the distinguishable layers (chondrium as well as the perichondrium) is also supported by the histological stain, Safranin O. This stain shows the presence of sulphated glycosaminoglycans in the chondrium layer of the construct, however these cartilage specific GAGs appeared not to be present at the periphery of the cartilage constructs, very similar to that of native tissue, shown in **Figure 3-2**. Elastic trichrome staining reveals that constructs lacked elastic whereas native shows an abundance of elastic fibers (highlighted by red arrow in **Figure 3-2**).

3.1.2.8 Biochemical Quantification

Biochemical analysis of native and engineered tissue supported the mechanical, histological, as well as immunohistochemical evaluations. Biochemical analysis (**Table 3-5**) showed that micromass constructs had on average 25% more DNA/mg of cartilage tissue weight, however this did not prove to be statistically significant ($P=0.086$). Proteoglycan (PG) levels between native tissue and engineered tissue were very similar, with engineered constructs showing 39.56% lower average of PG/mg of tissue, and 49.88% lower PG/DNA compared to native tissue ($P = 0.042$, $p=0.028$ respectively). Constructs showed 80.83%, lower levels of collagen/mg ($P = 0.016$) and 84.28% of collagen/DNA.

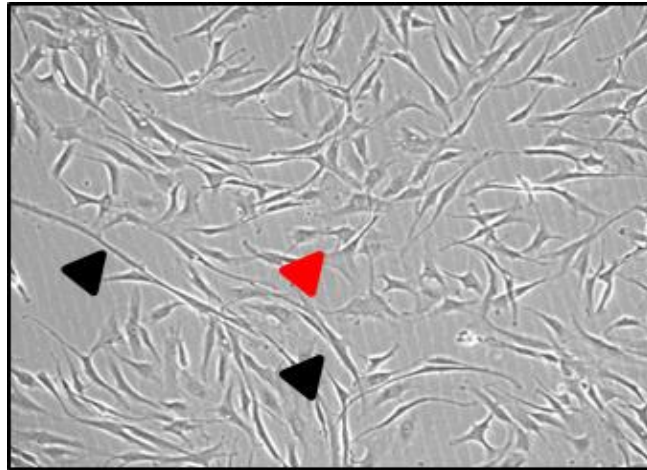


Figure 3-7. Primary auricular chondrocyte culture.

Auricular chondrocytes cultured *in vitro* monolayers show the presence of both rounded chondrocytes (red arrow) as well as spindle-shaped

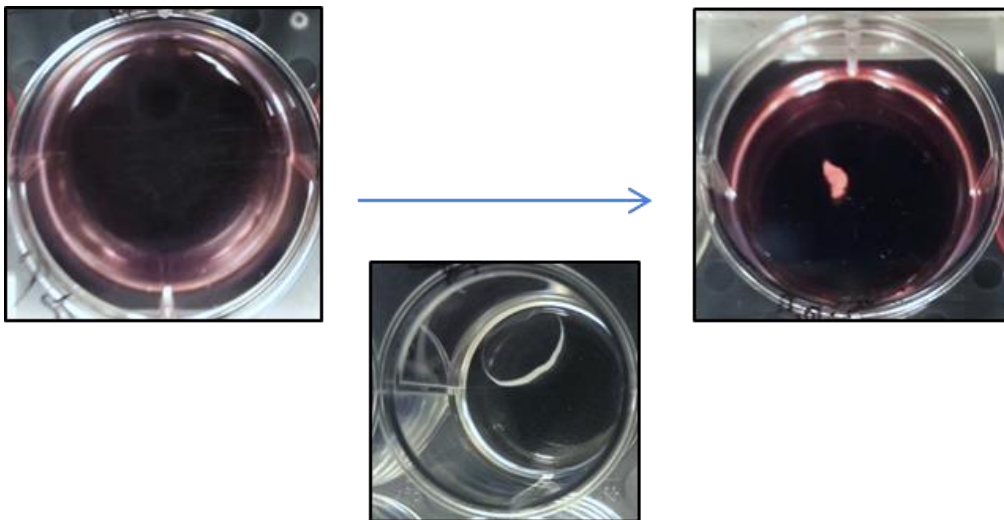


Figure 3-8. Paediatric auricular chondrocyte aggregation assay.

Passaged auricular chondrocyte monolayers aggregate upon treatment with chondrogenic stimuli (TGF- β 3). Passaged Human auricular chondrocytes show aggregation pattern similar to that observed in MSCs during early limb bud development.

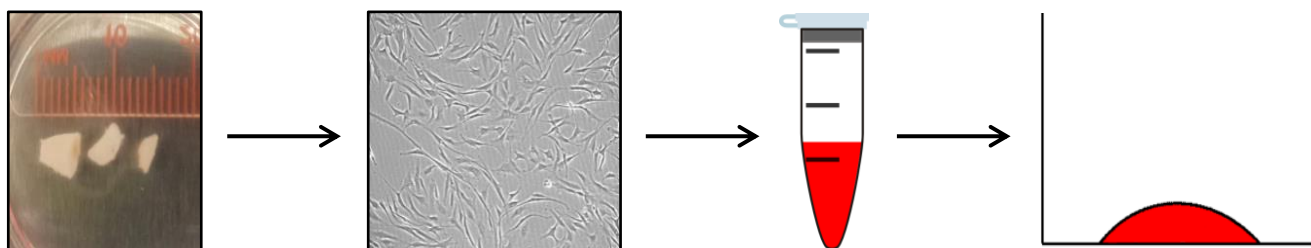


Figure 3-9. Cartoon depiction of micromass culture formation.

Chondrocyte harvest from surgical auricular tissue remnants, expanded *in vitro*, and cultured under micromass culture conditions.

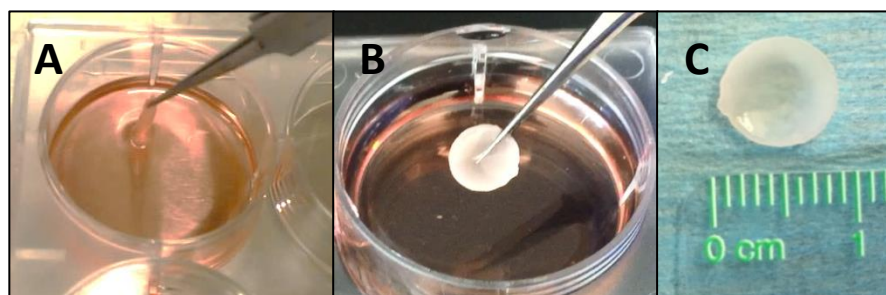


Figure 3-10. Macroscopic appearance of micromass cultures undergoing chondrogenesis at different stages of construct formation.

A) Micromass culture which has not undergone full chondrogenesis, B) micromas that has undergone chondrogenesis *in vitro*, which shows mechanical stability, C) representative size of constructs.

Table 3-4. Mechanical properties of paediatric scaffold-free auricular tissue constructs.

Condition	Thickness (mm)	Poisson's ratio	Bulk modulus (MPa)	Elastic modulus (MPa)
Native (n=2)	0.78 ± 0.00	0.31 ± 0.12	2.83 ± 1.17	7.21 ± 2.15
Constructs (n=4)	0.46 ± 0.06	0.47 ± 0.17	1.23 ± 0.74	3.60 ± 1.49

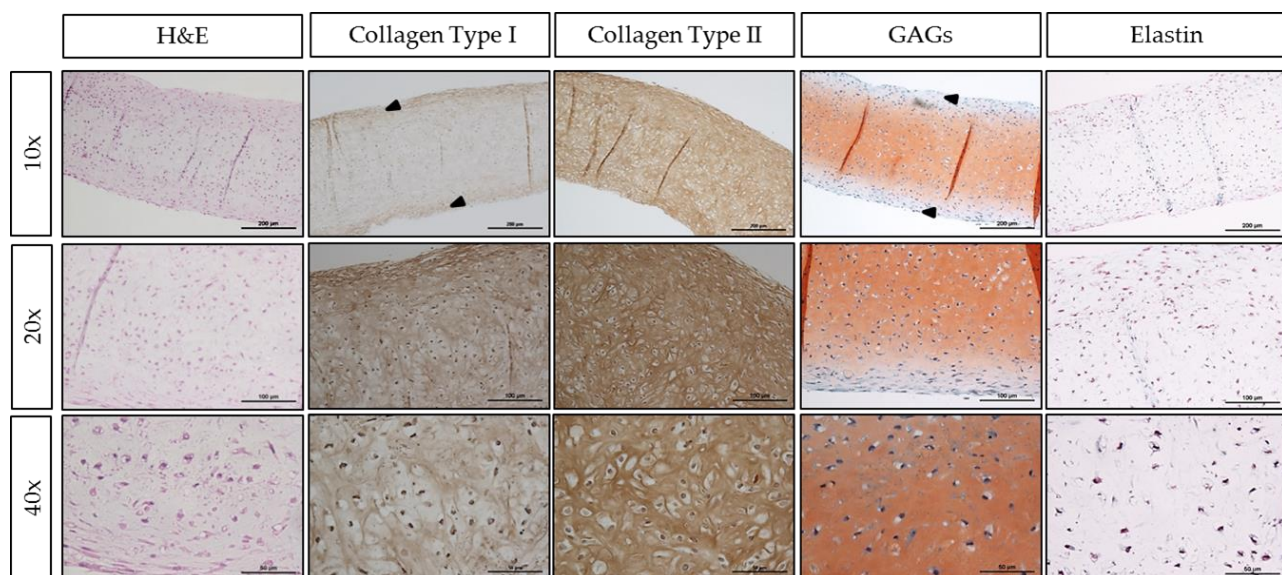


Figure 3-11. Histological and immunohistochemical examination of micromass engineered auricular tissue constructs show similarities to native paediatric auricular tissue. Histological staining for: H&E, for an overall structural morphology, Safranin O for GAGs (orange/red), Elastin trichrome for elastic fibers, and immunohistochemical analysis for Collagen type I, and Collagen type II.

Table 3-5. Biochemical properties of native and micromass engineered auricular cartilage constructs.

	Native (n=2)	Construct (n=5)
DNA (ug)	-	10.84 ± 2.38
DNA (ug/mg)	0.32 ± 0.01	0.38 ± 0.07
PG(ug)	-	567.47 ± 84.59
PG(ug/mg)	32.93 ± 1.23	19.9 ± 2.47
PG(ug/DNA)	104.43 ± 9.55	52.34 ± 7.32
OH(ug)	-	76.08 ± 10.44
OH(ug/mg)	13.93 ± 1.46	2.67 ± 1.34
OH(ug/DNA)	44.52 ± 5.32	7.01 ± 2.19

3.2 Discussion

Autologous hyaline cartilage grafts used to provide auricular grafts in reconstruction surgeries offer good aesthetics and long-term stability in the hands of experienced surgeons (Tollefson, 2006; Brent, 1999). However, such grafts often require large costal cartilage harvests, leaving significant donor-site morbidities, and limiting the minimum age for surgical intervention, and thus increasing the psychosocial effects of microtia on the child (Brent, 1999; Johns, Lucash, Im, & Lewin, 2015). Tissue engineering has been explored in providing an alternative. In this work, I addressed this issue by generating scaffold-free auricular cartilage constructs from small human paediatric tissue remnants.

In order to establish tissue engineering reference points, tragal and conchal bowl elastic cartilage remnants from cholesteatoma surgeries (**Table 3-2**) were analyzed mechanically, histologically, immunohistochemically, and biochemically (**Figure 3-2, Table 3-4, Table 3-5**). Although studies have identified differences in mechanical and biochemical properties of the different regions of the ear, the identified differences were quite small, allowing us to pool our data from the tragus and concha together (Nimeskern, et al., 2015). To identify the native thickness of mature auricular cartilage, whole ears were obtained, sectioned and analyzed. Thickness measurements of the elastic cartilage in these auricles revealed significant thickness variation between individuals as well as different regions of the ear varying from 0.65mm-1.85mm. Interestingly the cadaver of much younger age of death than other displayed significantly lower cartilage thickness. Studies have identified that thickness of auricular elastic cartilage increases with age, while GAG and collagen contents decrease, which has been reported to be due to the fragmentation of elastic fibers (Ito, et al., 2001; Nimeskern, et al., 2015). Importantly, although it has been shown that the elastic cartilage within the external ear enlarges with age, our thickness evaluations did not exceed measurements of 1.85mm, indicating that cartilage size increases could not be the only factor responsible for whole auricular tissue enlargement. Interestingly, as shown in the cadaveric tissue analysis (**Figure 3-3, Figure 3-4, Figure 3-5, Figure 3-6**), soft connective tissue surrounding the auricular elastic cartilage is multiple times larger in thickness than the auricular cartilage itself, and thus contributes to the overall auricular size much more than the auricular framework. Although most studies have identified that cartilage ECM degradation is a driving force in external ear size increase past maturity, we hypothesise that auricular soft tissue

size increases may have even a larger impact on whole enlargement with age than cartilage enlargement. Additionally, although it has been shown that differences in mechanical and biochemical properties exist in auricular cartilages from different age groups, auricular cartilage from children 20 and under are commonly treated as uniform (Ito, et al., 2001; Nimeskern, et al., 2015), and are ideal to use for auricular tissue engineering as older cartilage tissue begins to deteriorate and would in turn perhaps give rise to mechanically unstable constructs.

In order to generate cartilage construct ears, roughly a hundred million cells are required (Saadeh, et al., 1999). To obtain this large number of cells from small harvests, commonly a cell expansion stage is carried out. The most common way to perform this is by *in vitro* monolayer culture, and although process dedifferentiates the chondrocytes by decreasing chondrogenic marker expression (collagen type II, elastin, and aggrecan, and sox9) (Ishak, et al., 2011), these dedifferentiated cells are often capable of reverting back to their chondrogenic state by undergoing redifferentiation under specific chondrogenic conditions (Yanaga, Imai, Fujimoto, & Yanaga, 2009; Yanaga, Imai, Fujimoto, & Yanaga, 2009). Interestingly, it has even been reported that adult dedifferentiated chondrocytes are multipotent, as they have been shown to express nearly identical surface marker expression profile as human MSCs, and multilineage differentiation capabilities, perhaps explaining their redifferentiation capabilities (de la Fuente, et al., 2004). To assess the response of our expanded auricular chondrocytes to chondrogenic stimuli, monolayers of expanded (Passage 3) paediatric auricular chondrocytes were analyzed. Aggregation of monolayer expanded paediatric auricular chondrocytes upon exposure to chondrogenic stimuli indicates a chondrogenic phenotype, such as mesenchymal condensation in skeletal development and chondrogenesis (Leonard, et al., 1991; Boeuf & Richter, 2010). The aggregation of these chondrocyte precursors in early development (**Figure 1-2**) via exposure to chondrogenic factors such as TGF- β 1, create a high cell density mass, which is thought to further induce chondrogenesis of these cells(**Figure 3-8**) (Boeuf & Richter, 2010). The requirement of high cellular density of expanded auricular chondrocytes was translated into our tissue engineering approach by the use of the micromass culture system. In this system, a large amount of cells is seeded onto a very small surface area (2×10^6 cells/50ul droplet), creating an *in vitro* multilayer of cells. Inducing these micromass culture systems via potent chondrogenic stimuli (10 ng/ml TGF- β 3, 0.1 μ M dexamethasone, 50 μ g/ml ascorbic acid 2-phosphat) for a prolonged period of time (8 weeks) enhanced their chondrogenesis to an extent which resulted in

mechanically stable tissue constructs **Figure 3-10**. Even though the pellet culture would also be able to create dense culture conditions, it would not have the potential to generate clinically relevant shapes of auricular tissue. Our observation that prolonged period of chondrogenic inductions are required for tissue constructs to mature has also been reported in the articular cartilage tissue engineering field, where reports have been made that culture periods of > 8 weeks is required to mature engineered cartilage tissue prior to implantation (O'Connell, et al., 2015).

Our micromass scaffold-free constructs showed mechanical stability and shape retention (**Figure 3-10**), and constructs approached 58% of native auricular tissue thickness with 8 weeks of *in vitro* static culture conditions. As seen in **Figure 3-11**, histological and immunohistochemical analysis revealed that the engineered construct shows the presence of a cartilaginous zone composed of chondrocytes residing in lacunae, surrounded by high levels of interterritorial matrix GAGs, collagen type II, and low levels of collagen type I in, very similar to that of native paediatric auricular cartilage seen in **Figure 3-2**. The presence of collagen type X was not examined, however studies have shown that tissue engineered auricular constructs in fact stain positive for this collagen (Gilpin, Weidenbecher, & Dennis, 2010) possibly due to the use of ascorbic acid that is provided to aid collagen fiber deposition (Leboy, et al., 1989). Interestingly, surrounding the cartilaginous zone, the tissue construct shows the presence of a fibrous perichondrium that is differentially rich in collagen type I content and is present on both sides of the cartilaginous zone, very similar to that of native tissue. Rarely found in tissue engineered cartilage, the presence of a perichondrium surrounding the cartilaginous zone is very encouraging as it is an important for successful construct implantation (Giardini-Rosa, et al., 2014; Yanaga, Imai, Fujimoto, & Yanaga, 2009; Yanaga, Imai, Koga, & Yanaga, 2012). However, although constructs exhibited good mechanical strength, they were not on par with native cartilage. This is supported by biochemical quantification (results shown in **Table 3-5**) where constructs contain 50-60% GAG content, and 15-19% of collagen content, to that of native tissue. To address this issue, additional culture conditions could be utilized to enhance chondrogenesis, and ultimately improving mechanical and biochemical parameters, as described in chapter 4. However importantly, our micromass constructs generated using TGF- β 1, TGF- β 3, IGF-1 chondrogenic factors were not significantly different (data not shown).

As seen in **Figure 3-11**, the micromass engineered tissue does not show the presence of elastic fibers, depicted by the lack of elastic trichrome, black fiber staining. The lack of elastic fiber deposition in *in vitro* tissue engineering is common, and is not limited to the field of auricular tissue engineering (Byers, Mauck, Chiang, & Tuan, 2008; Giardini-Rosa, et al., 2014; Hinderer, et al., 2015). Additionally, to investigate the elastin expression in expanded auricular chondrocyte cultures, a western blot probing for elastin (antibody: ab23747, antigen: full length native protein purified from human skin) was performed. Interestingly, results indicated that elastin was present in auricular chondrocyte cultures in the presence of auricular chondrogenic stimuli, even though elastic fibers were still not observed in cartilage constructs. Functional elastic fiber deposition is a major challenge in the field of elastic tissue engineering, as tropoelastin is commonly readily detected in cultures and tissue-engineered constructs (Ishak, et al., 2011; Hinderer, et al., 2015; Giardini-Rosa, et al., 2014).

This indicates that the in fact the absence of elastic fibers in tissue constructs is not due to absence of tropoelastin, but perhaps in other players involved in elastogenesis, such as elastin binding protein (EBP), and lysyl oxidase (LOX), which are proteins responsible for exporting tropoelastin to extracellular space, and crosslinking it onto fibrillin fibers respectively. It is important to note, that this problem seems to solely exist for *in vitro* engineered tissue constructs, as groups who have matured scaffold-free paediatric auricular cartilage constructs subcutaneously, have observed the presence of functional elastic fibers (Yanaga, Imai, Koga, & Yanaga, 2012). In agreement with this, studies have identified that elastin expression decreases dramatically with *in vitro* expansion of auricular chondrocytes (Ishak, et al., 2011). Therefore, it is apparent that gaps exist for *in vitro* auricular cartilage tissue engineering that has yet to be identified and utilized to enhance and mature constructs *in vitro* prior to patient implantation.

Studies have identified that the native mRNA of tropoelastin expression in auricular chondrocytes systematically decreases with common monolayer expansion (Ishak, et al., 2011). To battle this, studies have expanded porcine chondrocytes in aggregate cultures rather than monolayer culture have, and have observed that these chondrocytes maintain their elastogenic ECM deposition *in vitro* (de Chalain, Phillips, & Hinek, 1999). Most likely, elastogenic expression profile is eliminated during dedifferentiation of chondrocytes through cellular attachment and expansion in monolayer culture, and is not reinitiated properly with the current standard of

elastic chondrogenesis differentiation cocktail , leading to cartilage constructs lacking elastic fiber deposition (de Chalain, Phillips, & Hinek, 1999). Preventing dedifferentiation of expanding chondrocytes by adopting aggregate culture expansion may preserve the elastogenic properties of auricular chondrocytes and lead to scaffold-free human paediatric auricular cartilage constructs, however to test this hypothesis, further experimentation is required.

Therefore in this chapter, I demonstrated a novel mechanism of generating auricular cartilage constructs using human paediatric auricular chondrocytes cultured in micromass culture systems, which resemble their native counterparts. This chondrogenic potential of paediatric auricular chondrocytes promises great potential for the clinical translation for the field of auricular tissue engineering by promising the possibility of generating scaffold-free auricular constructs *in vitro*. In future studies, we propose to carry out *in vitro* aggregate culture expansion phase prior to micromass culture construct formation in hopes of preserving the elastogenic phenotype of paediatric auricular chondrocyte scaffold-free culture system. Additionally xenograft implantation experiments to assess the *in vivo* stability of these auricular cartilage constructs as well as elastogenesis progression as observed in other subcutaneous auricular cartilage constructs.

Chapter 4

Rotating Wall Vessel Bioreactor (Micro Gravity Bioreactor)

4.1 Introduction

Developing functional tissue *in vitro* often requires systems supporting biologically active environments called bioreactors. Bioreactors are commonly used for tissue engineering purposes because tissue generation usually requires constant replenishment of nutrients and removal of toxic waste, since cellular density is much higher than monolayers cultures (Freed & Vunjak-Novakovic, 2000; Martin, Wendt, & Heberer, 2004). Rotary Cell Culture System (RCCS) utilizing a Slow Turning Lateral Vessel (STLV, shown in **Figure 4-1**) provides a means of 3-D culture in which the medium and the construct are in constant rotational motion, mimicking a free floating (microgravity) condition, predicted to promote construct growth and development (Yu, et al., 2011). This system is useful in auricular tissue generation because it allows for the free floating of complex 3D masses.

Although micromass culture cartilage constructs showed promising physical properties, it would be ideal to enhance them even more so to ensure construct shape retention post-implantation. To address this issue, I investigated the effects of the RCCS in hopes of enhancing the physical properties of the constructs.

Due to the scarcity of available normal auricular cartilage, preliminary experiments were run using human paediatric nasal chondrocytes, which differs from auricular chondrocytes in that their tissue of origin is hyaline cartilage, rather than elastic cartilage. In order to assess the effects of the micro gravity bioreactor, two separate conditions were created to cover both with-carried and without carrier methods of chondrogenesis. Therefore, two sets of experiments were performed. 1) Nasal chondrocytes were embedded in a collagen type I gel, and 2) Scaffold-free generation of nasal chondrocyte multilayers.

4.2 Materials and Methods

4.2.1 Human Paediatric Nasoseptal Chondrocyte harvest and Isolation

This study was approved by the Hospital for Sick Children Research Ethics Board (REB 1000039790). From December 2013 to April 2015, a total of 8 usable nasoseptal cartilage samples were obtained from the surgical staff at the Hospital for Sick Children under, for which the demographic is shown in **Table 4-1**. Samples varied not only from patient to patient in terms of gender and age, but also by anatomical resection sites. Thus the cohort permitted trialing the heterogeneity represented in the paediatric population.

all soft tissue was removed, and the cartilage was washed three times with PBS containing 1X antibiotic/antimycotic. Washed cartilage was then minced into smaller pieces of roughly 5mm x 5mm, to increase surface area for digestion. These cartilage pieces were then subjected to the same digestion and culture methods as the earlier described auricular chondrocytes, section 3.1.1.5.

Interestingly, unlike auricular chondrocyte primary culture, nasal chondrocytes exhibit a much more uniform chondrocyte culture **Figure 4-2**, with virtually no fibroblast like cells present, whereas auricular chondrocyte primary cultures showed relatively abundant presence of such cells, shown in **Figure 3-7**.

4.2.2 Collagen embedded nasal chondrocyte culture

2×10^6 Passage 3 nasal chondrocytes were embedded in neutralized 5mg/ml rat tail type I collagen (R&D Systems) , and plated on silicone coated non-adherent plates as a 100 μ l droplet, and allowed to settle for 2 hours. Upon settling, 2 ml of medium containing DMEM/F12 supplemented with 10% FBS was added to each well, and cells were allowed to fully attach to the collagen gel overnight at 37°C and 5% CO₂. Three collagen embedded samples were then grown under static culture, and three were placed in the RCCS, both in chondrogenic medium. Cultures were then obtained after 30 days of culture, and subjected to histological, immunohistochemical, biochemical, and mechanical examination.

4.2.3 Scaffold-free nasoseptal cartilage tissue engineering construct

Freshly isolated nasal chondrocytes (passage 0 cells) were split into two different treatment groups. 1) An induction group in which the cells were induced towards retaining their chondrogenic phenotype via treatment with chondrogenic mediums, and 2) An expansion group in which the cells were expanded. 1×10^5 freshly isolated chondrocytes were plated in monolayer in a 6 well plate, and the remaining half of the cells were plated at a seeding density of 5×10^3 cells/cm² 5 flasks for expansion (for each patient sample). Upon confluency of the expanding cells, 7×10^5 cells were added on top of the induction group, creating a multilayer culture system, and the remaining 3×10^5 cells were passaged, and further expanded. In total, four sequential layering's were performed, generating multilayers consisting of P0 cells, P1, P2, and P3 cells respectively. This cellular layering technique also employed by another group, has been shown to have a positive impact on chondrogenesis (Yanaga, Imai, Koga, & Yanaga, 2012). After 4 weeks of continuous layering, the multilayer was cut in half using a sharp scalpel. One half was kept in the plate well under continuing static chondrogenic culture conditions, and the other half was moved into the bioreactor and exposed to micro gravity 3D culture conditions in the same chondrogenic medium as those in static culture. Results are shown in Figure 4-3.

Table 4-1. Human paediatric nasal cartilage sample demographic.

Sample #	Age	Gender	Tissue Location
1	13	M	Septum
2	9	M	Septum
3	13	F	Septum
4	7	M	Septum
5	14	M	Septum
6	6	M	Septum
7	11	F	Septum
8	11	M	Septum



Figure 4-1. Rotary Cell Culture System (RCCS) utilizing a Slow Turning Lateral Vessel (STLV) (Synthecon Inc.)

4.3 Results

4.3.1 Collagen embedded nasal chondrocyte culture

Chondrocyte embedded collagen type I constructs showed an average weight of 49.25mg, whereas static constructs showed an average of 32.55mg, and the difference proved to be significant ($P=0.023$). Bioreactor constructs showed an average thickness of 2.43 mm, whereas static chondrocytes showed an average thickness of 2.81 mm, however this difference did not prove to be significant ($P= 0.189$). Both bioreactor and static culture conditions generated constructs with high Poisson's ratio (bioreactor = 0.409, and Static = 0.5). However, the bioreactor constructs showed a lower average than static cultures, more closely resembling that of native tissue.. Both conditions yielded constructs that had significantly lower Bulk modulus, however, constructs generated in the bioreactor showed a higher bulk modulus than those grown in static culture, indicating the generation of stronger tissue. Lastly, both conditions again yielded tissue with much higher elastic moduli than native cartilage, however a comparison between bioreactor and static culture conditions, it was identified that constructs grown in the bioreactor had a higher elastic modulus, however, this difference was not significant ($P=0.18$). Over all mechanical testing results show that both sets of constructs show very different mechanical properties compared to native nasal cartilage tissue, however, results seem to slightly favour bioreactor generated constructs over those of static culture.

4.3.2 Scaffold-free nasoseptal cartilage tissue engineering

Unlike collagen embedded chondrocyte constructs, there was a significant difference between bioreactor and static culture conditions for nasoseptal chondrocyte multilayers. Multilayer cultures induced towards chondrogenesis in the bioreactor developed into three dimensional, mechanically stable cartilaginous constructs, whereas static culture multilayers remained quite fragile, and did not reach a point of mechanical stability. Bioreactor constructs showed qualitative cartilage characteristics, such as glossy, white, and opaque phenotypes, whereas static cultures did not (**Figure 4-4**). Bioreactor constructs retained their shape completely upon handling, whereas static culture exhibited inadequate mechanical strength and did not retain its shape upon handling with tweezers.

Bioreactor constructs averaged 0.9 ± 0.2 mm in thickness, whereas halves differentiated under static conditions are only 0.23 ± 0.07 mm in thickness, a 4 fold difference compared to engineered construct thickness. Interestingly, histological and immunohistochemical evaluations revealed that both groups of constructs (bioreactor and static), show high levels of collagen type II, and low levels of collagen type I, as expected in cartilage tissue. However, only constructs grown in micro gravity bioreactor (stain positive with safranin O and) show the presence of GAGs (**Figure 4-4**). These histological observations are also supported by biochemical quantification assays of ECM components (**Table 4-4**) which indicate that carrier free chondrocyte monolayers cultured in the RCCS were the only construct/culture condition which resulted in constructs that exhibited similar GAG and collagen levels to native cartilage (52.55% similarity for GAGs, and 17.4% for collagen).

4.3.3 Statistical Analyses

All data were expressed as mean \pm standard error of the mean (SEM). Samples from different patients were not mixed at any point in the study. Student's t-test was used to compare data, and p-values less than 0.05 were considered significant. Statistical tests were performed using Microsoft Excel 2010 software for Windows.

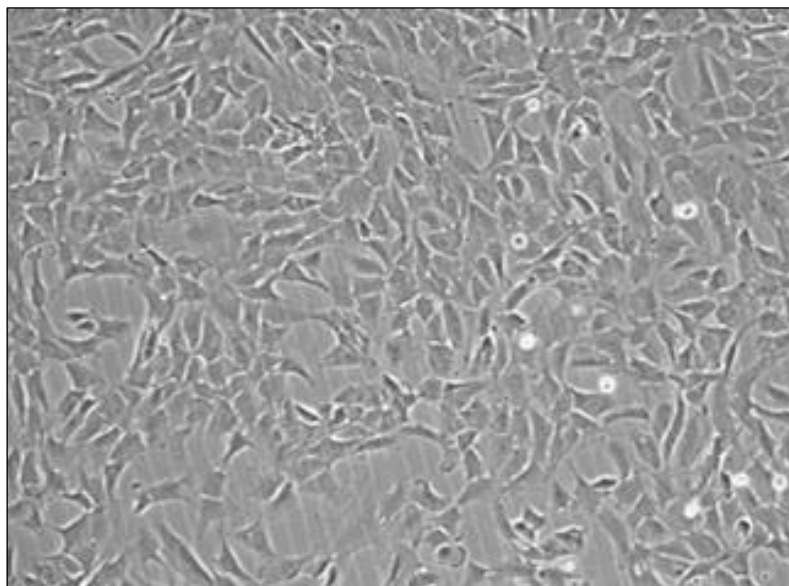


Figure 4-2. Paediatric nasoseptal chondrocytes primary cultures.

Nasoseptal primary chondrocyte culture exhibits uniform culture of round shaped chondrocytic cells. 10x Magnification.

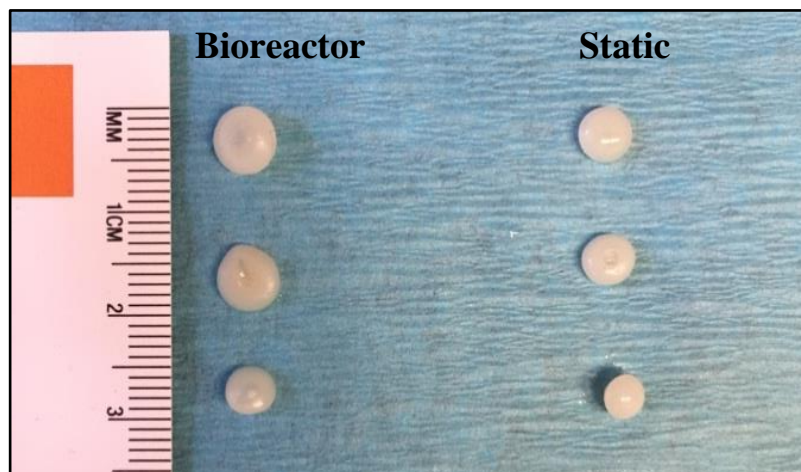


Figure 4-3. Macroscopic appearance of bioreactor vs. static culture generated nasoseptal constructs using a collagen type I gel. Passage 3 nasoseptal chondrocytes embedded in collagen type I gel and treated for 30 days.

Table 4-2. Mechanical properties of native nasoseptal cartilage, bioreactor and static culture generated using collagen type I hydrogels.

Sample	Condition	Thickness (mm)	Poisson's ratio	Bulk modulus (MPa)	Elastic modulus (MPa)
-	Native (n=4)	1.56 ± 0.05	0.32 ± 0.18	12.46 ± 9.2	29.88 ± 14.24
C+C	Bioreactor (n=3)	2.43 ± 0.13	0.41 ± 0.015	1.23 ± 0.86	3.27 ± 1.22
C+C	Static (n=3)	2.81 ± 0.16	0.50 ± 0.00	0.35 ± 0.16	1.07 ± 0.15

Table 4-3. Mechanical properties of paediatric native nasoseptal cartilage, bioreactor, and static culture scaffold-free tissue constructs.

Sample	Condition	Thickness (mm)	Poisson's ratio	Bulk modulus (MPa)	Elastic modulus (MPa)
-	Native (n=4)	1.56 ± 0.05	0.32 ± 0.18	12.46 ± 9.20	29.88 ± 14.24
C	Bioreactor (n=3)	0.90 ± 0.12	0.23 ± 0.05	8.05 ± 1.27	19.78 ± 3.12
C	Static (n=3)	0.23 ± 0.05	0.50 ± 0.00	0.25 ± 0.12	0.77 ± 0.32

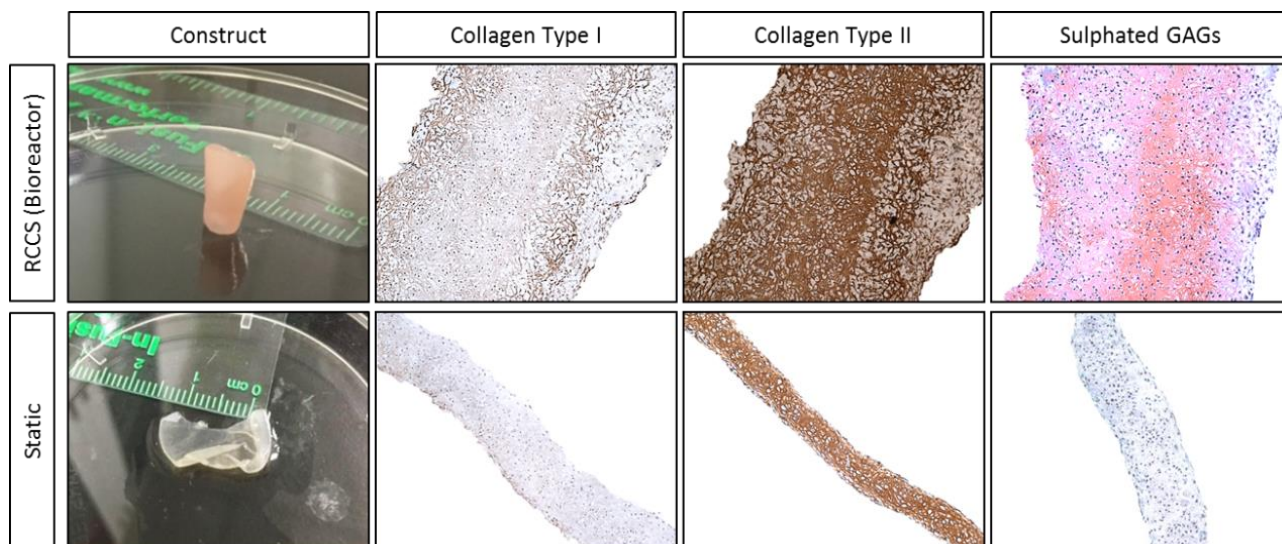


Figure 4-4. Macroscopic appearance of scaffold-free engineered nasoseptal tissue constructs along with their respective histological and immunohistochemical evaluations.

Table 4-4. Biochemical properties of nasoseptal native cartilage, bioreactor, and static culture scaffold-free tissue constructs.

	Bioreactor			Static	
	Native	Cells + Collagen	Cells	Cells + Collagen	Cells
	N=4	N=3	N=3	N=3	N=3
DNA Total (μg)	-	12.07 ± 2.51	4.22 ± 0.65	11.27 ± 4.59	2.94 ± 1.20
DNA/ $(\mu\text{g}/\text{mg})$	0.19 ± 0.03	0.23 ± 0.02	0.21 ± 0.02	0.28 ± 0.06	0.26 ± 0.03
GAG	-	651.38 ± 173.15	612.92 ± 86.18	485.23 ± 55.75	154.15 ± 24.40
GAG(μg)/mg	54.01 ± 5.14	12.41 ± 0.52	14.94 ± 1.87	13.32 ± 3.74	9.67 ± 2.1
GAG/DNA	276.35 ± 40.72	53.52 ± 6.17	145.24 ± 12.45	43.03 ± 16.24	52.43 ± 8.43
Collagen	-	43.62 ± 10.35	26.11 ± 4.23	28.89 ± 1.46	16.013 ± 4.22
Collagen(μg)/mg	6.76 ± 1.14	0.84 ± 0.03	0.63 ± 0.02	0.77 ± 0.10	1.09 ± 0.32
Collagen/DNA	35.47 ± 5.32	3.62 ± 0.85	6.18 ± 1.44	2.56 ± 1.39	5.44 ± 1.86

4.4 Discussion

Bioengineering full thickness tissue constructs *in vitro* static culture is challenging due to limited nutrient delivery and toxic waste removal. Bioreactors have been designed to provide a solution to this challenge in the field by providing constant replenishment of medium. In this work I aimed to investigate the effects of RCCS bioreactor chondrogenic conditions on nasal chondrocyte multilayers in hopes to identify a method for generating mechanically stable scaffold-free human cartilage constructs *in vitro*.

To assess the condition specific effects of the RCCS culture conditions on nasoseptal cartilage construct generation, two conditions were tested. In one method, expanded chondrocytes were seeded in collagen type I hydrogel, and in the other expanded nasoseptal chondrocytes were used to generate a multilayer consisting of 4 layers, without the use of any scaffolds/carriers. Interestingly, the effect of the RCCS bioreactor varied between these two conditions. Constructs that were embedded in collagen type I gels were indifferent to bioreactor stimulation identified via overall morphological appearance analysis (**Figure 4-3**) mechanical properties (**Table 4-2**) and biochemical properties (**Table 4-4**), whereas carrier free constructs significantly benefitted from microgravity simulation. Bioreactor stimulated scaffold-free constructs developed into much thicker (0.9mm vs. 0.23mm, 4 fold difference) and more mature cartilage tissue constructs expressing higher levels of Collagen type II and GAGs, whereas those grown in static culture remained fragile and did not express any sulphated GAGs (**Figure 4-4** indicating safranin-O staining, and **Table 4-4** showing significantly lower collagen and GAG levels detected by biochemical assay). These morphological and histological differences are also supported by the improved mechanical properties of the bioreactor stimulated multilayer in contrast to static culture, as shown in **Table 4-3**.

Generating multilayers of expanded human paediatric auricular chondrocytes *in vitro* has been shown to facilitate their redifferentiation *in vivo* (Yanaga, Imai, Koga, & Yanaga, 2012) (Yanaga, Imai, Fujimoto, & Yanaga, 2009). In our approach, nasoseptal multilayers were placed into the bioreactor culture instead, bypassing a high cell density mass formation as described by Yanaga et al. (2009, 2012). Revealed by histological and immunohistochemical analysis (**Figure 4-4**), cells cultured in the RCCS continued to expand, resulting in constructs that consist of more

cellular layers than those cultured in static conditions. Bioreactor environment which allows the survival of higher cell densities is what we account to the production of much higher levels of sulphated GAGs, as it has been shown that GAG production can be increased by increasing cellular densities (Kobayashi, Meir, & Urban, 2008). Although Authors claimed that this was a difficult challenge as increasing cell densities could induce extreme hypoxia in the centre of the 3D construct, leading to apoptosis and debilitating ECM production. However, generating dense multilayers within the bioreactor ensures that the cell density is increased while cellular health and viability is maintained, ultimately yielding thicker and more mechanically stable constructs.

As described in section 4.2.3 each set of bioreactor vs. static culture experiment was initiated using two halves obtained from cutting one multilayer mass into two sections, each pair of bioreactor vs. static culture constructs were therefore identical in cellular identity and cellular density. Thus the differences that are observed between scaffold-free constructs can be solely attributed to RCCS culture conditions, and not to differences in cellular composition or density differences. Interestingly, nasal chondrocytes embedded in collagen type I were not able to distinguish RCCS and static culture systems, unlike scaffold-free constructs. We suggest that their chondrogenesis is not inhibited by the use of collagen type I hydrogel, since groups have achieved cartilage construct generation using collagen type I gels as chondrocyte carriers (Reiffel, et al., 2013), and articular chondrocyte matrix production and turnover has been shown to be promoted by collagen type I coated inserts (Rutgers, et al., 2013). However, further experimentation is needed to determine if collagen type I may actually interfere with bioreactor stimulated chondrogenesis of human nasal chondrocytes specifically. Interestingly, previous studies focusing on identifying the effects of the RCCS on chondrogenesis have reported inconsistent results. Vunjak-Novakovic et al. (1999) showed that calf articular chondrocytes seeded in fibrous polyglycolic acid scaffolds generated much higher amounts of GAGs, collagens, equilibrium modulus, and dynamic stiffness in RCCS culture conditions compared to static cultures. However, Mayer-Wagner et al. (2014) showed that human mesenchymal stem cell pellet cultures differentiated under bioreactor culture conditions showed significantly reduced safranin-O and collagen type II staining compared to static culture conditions. Considering the varying effects of the RCCS on different cell types, from our data we conclude that RCCS seems to be rather selective on construct generation conditions.

Additionally, there have been many groups that have focused on optimizing the chondrogenic medium for both auricular and nasoseptal cartilage construct formation. In this study, because I was particularly interested in investigating the effects of the RCCS, the chondrogenic medium was kept quite simple in terms of composition (DMEM/F12 supplemented with 1x ITS premix, 10 ng/ml TGF-B1, 0.1 μ M, Dexamethasone, 50 mg/ml ascorbic acid, and 1x antibiotic/antimycotic mix). It remains to be explored whether the addition of such factors in conjunction with the use of the RCCS may further enhance chondrogenic differentiation in such culture systems.

In this study, I investigated the effects of RCCS on human nasoseptal chondrogenesis. My results indicate that RCCS enhances chondrogenesis only of carrier-free chondrocyte multilayers, and is able to thicken and strengthen fragile chondrocyte multilayers by permitting the growth of additional layers via outward expansion. Exploiting this potential, we hypothesize that auricular scaffold-free cartilage constructs generated *in vitro* under static conditions could be further matured in the RCCS bioreactor, enhancing cartilage tissue formation. This is crucial, because although micromass auricular cartilage constructs portrayed characteristic cartilaginous properties (covered in chapter 3), they only matured to 0.49 mm in thickness, whereas native auricular tissue measured from 0.63-1.74 mm. This highlights the demand of having the means of generating thicker auricular tissue. Based on our results, the RCCS provides a platform, regulating construct thickness of auricular tissue that would be required for patient specific clinical applications, supported by **Figure 3-1**.

Chapter 5

Conclusions and Future Directions

5.1 Conclusions

Extreme spatial complexity of the external ear has limited auricular tissue regeneration methods to those which incorporate scaffolds. Although utilizing scaffolds has been beneficial for construct shape specificity, it has been detrimental for construct shape retention and stability over prolonged periods of time. To address this concern, scaffold-free tissue engineering is being explored. In this work I obtained human paediatric auricular cartilage samples and established normal tissue endpoints comprising of mechanical, histological, immunohistochemical, and biochemical components. Additionally, I established culture of chondrocytes harvested from such tissues *in vitro* and utilized micromass culture technology to generate scaffold-free auricular cartilage constructs *in vitro*, which resembled established endpoints.

In conjunction, static culture limited cartilage tissue generation to $0.46\text{mm} \pm 0.06$ in thickness. However, upon native tissue examination **Table 3-3**, it became readily apparent that auricular cartilage varies in thickness in the auricle, igniting the search for possible ways to increase tissue thickness. Bioreactors have long been thought to enhance tissue generation *ex vivo*, thus I investigated the effect of RCCS on cartilage construct formation. Although nasoseptal chondrocytes were used rather than auricular chondrocytes, my results indicated that nasoseptal chondrocyte multilayers consisting of only four layers differentiated in the RCCS generate cartilage tissue constructs measuring an average of $0.9\text{mm} \pm 0.12$ in thickness and express cartilage specific ECM markers, both of which were not observed in constructs kept in static conditions. We propose that auricular micromass constructs could be further matured to resemble native tissue to a higher extent via additional culture time within the RCCS.

In this work, we report the first case of *in vitro* human paediatric scaffold-free auricular cartilage tissue engineering. Developing tissue constructs without the incorporation of scaffolds is a promising strategy as it ensures maximum patient compatibility with reduced post-surgical complications and construct stability. Although our methods show great promise in the field of

human auricular tissue as well as nasoseptal cartilage tissue engineering, future studies need to be carried out exploiting complex 3D structures and long term *in vivo* stability of such constructs.

5.2 Future Directions

Micromass cartilage constructs generated on the flat surface of a 6-well plate are only a showcase of the potential of this culture system. To be able to provide surgeons a cartilage construct that they deem worthy of implantation, demands the cartilage construct to recapitulate the patient specific shape, ultimately eliminating the requirement for surgeons to hand carve each pinnae.

In order to be able to generate anatomically distinct auricular shaped tissue, patient specific molds need to be generated. This 3-D mold would then replace the flat surface of the well, providing the micromass culture with anatomical information. Since over 75% of microtia cases are unilateral, a 3D scan of the counter lateral auricle can be used to generate a digital 3D copy of a normal auricle for the affected side using computer software such as Cyberware Rapid 3D Digitizer (3030 Digitizer, Monterey, CA) as shown by (Reiffel, et al., 2013). This digital copy of the patients' ear can be converted to a 3D printer file and ultimately 3D printed using a biocompatible composite mold that could then be used for the base of the micromass culture, replacing the flat 6-well plate used in this study. It is important to note that this 3D printed construct will not be incorporated into the micromass construct, as the micromass construct will be peeled off and separated from this mold once it has undergone sufficient levels of ECM production and chondrogenesis, as it did so from the plate. Studies have shown that methods which generate flat constructs can be successfully translated to 3D printed molds (Rosa, et al., 2014). The mold would be printed in a cylindrical form, such that it would fit into regular 6-well plates that are were used in our study. Commonly, cell attachments to such 3D printed synthetic molds are not efficient, and they need to be coated. Commonly groups have used animal derived collagens; however we propose a more clinically relevant type of coating, such a patient specific fibronectin, which has been shown to be secreted by chondrocytes *in vitro* as well (Dessau, Sasse, Timpl, Jilek, & von der Mark, 1978).

Auricular scaffold-free micromass constructs grown in static culture conditions show comparatively close mechanical, histological, immunohistochemical, and biochemical properties compared to native tissue. However, native auricular cartilage varies in thickness, and for distinct anatomical regions of the external ear, there may be a need to generate constructs thicker than the 0.49mm that were generated in this study. We predict that layering more cells in the micromass culture will not be helpful, as nutrients will not be easily delivered to the most inner cells through diffusion and toxic cellular waste will accumulate and cells will begin to die. A potential remedy would be to place the partially developed tissue into a bioreactor such as the RCCS described in Chapter 4 of this work. We tested this concept using nasoseptal chondrocytes, and exceptional results were achieved, outlined in section 59. Multilayers containing only 4 layers of chondrocytes were placed within the bioreactor became nearly 4 fold thicker than tissue in static culture, and developed into tissue that showed much higher mechanical strength, as well as increased histological, immunohistochemical, and biochemical similarities to native tissue. Therefore, we believe that to further mature auricular micromass constructs into thicker cartilage constructs, micromass cartilage constructs can be enlarged and matured in the RCCS.

The final stage of this project would be to test the feasibility and long term stability of scaffold-free auricular cartilage tissue constructs *in-vivo*, via a xenografting approach by implanting constructs into nude mice or rats. This is a very important step in moving scaffold-free tissue engineering forward, as it will provide information on how constructs would behave with the patient post-surgery. Interestingly, even though our *in-vitro* constructs did not show the presence of elastic fibers, it has been shown that once constructs are grafted subcutaneously into animal models, the tissue matures into an elastic cartilage constructs, and develops the abundant elastin assembly (Reiffel, et al., 2013) (de Chalain, Phillips, & Hinek, Bioengineering of elastic cartilage with aggregated porcine and human auricular chondrocytes and hydrogels containing alginate, collagen, and kappa-elastin., 1999). Maturing auricular implants in patients has also been investigated by Yanaga H (2012), which results indicating that tissue matured well *in vivo*, and aside from enhancing chondrogenesis and driving it towards completion, it also induces functional elastic fiber assembly. Therefore, potentially in the future, micromass generated scaffold-free auricular cartilaginous constructs, matured in the RCCS, can be implanted at the site of interest, and allowed to complete its maturation process as well as undergo elastogenesis,

and vascularization to successfully create a patient specific auricular construct, with very minimal morbidity and surgical difficulties.

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