Co-culture of primary chondrocytes and stem cells for regeneration of cartilage in the head and neck

A thesis submitted in fulfilment of the requirements for the degree of Master of Philosophy

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Statement of Originality

I declare that the contents of this thesis are comprised of my own original work. This thesis has not been submitted, either in whole or in part, for any other degree or professional qualification at another institution. The intellectual content of this thesis has been produced through my own work, with all contributions by other authors clearly stated and cited in line.

Authorship Attribution Statement

Chapter 1:

This introductory chapter was researched and drafted by me in its entirety. All diagrams were created by me for exclusive use in this manuscript and its contained publications.

Chapter 2:

I designed and conducted the protocol for the systematic review, interpreted the data and drafted the manuscript.

Protocol design was aided by my supervisors A/Prof Daniel Steffens and Payal Mukherjee.

Preparation of the final manuscript was aided by the collaborative efforts of A/Prof Daniel Steffens, Dr Johnson HY Chung, Dr Steven Posniak, Mr Kai Cheng, Prof Jonathan Clark, Prof Gordon Wallace and A/Prof Payal Mukherjee.

This review is under review for publication with Cells Tissues Organs. The findings of this review have been presented at the Australian Society of Otolaryngology Head and Neck Surgery 71st Annual Scientific Meeting (17 – 19th September 2021) and the Australian Biomedical Engineering Conference (21st – 22nd October 2021).

Chapter 3:

I designed and conducted the protocol for the animal study, interpreted the data and drafted the manuscript.

Protocol design and data collection were aided by my supervisor A/Prof Payal Mukherjee and Dr DS Abdullah Al Maruf, Dr Steven Posniak, Dr Johnson HY Chung, and Dr Xiao Liu in collaboration with the Intelligent Polymer Research Institute, University of Wollongong. I designed the cartilage harvest protocol which I conducted at the Charles Perkins Centre under

the supervision of A/Prof Payal Mukherjee. I conducted the cell digestion with Dr DS Abdullah Al Maruf at the RPA Institute of Academic Surgery with further digestion performed by Dr Steven Posniak and Dr Johnson Chung at the Intelligent Polymer Research Institute, University of Wollongong. The cell proliferation was performed by Dr Steven Posniak, Dr Johnson Chung and Dr Xiao Liu at the Intelligent Polymer Research Institute, University of Wollongong. Preparation of the final manuscript was aided by the collaborative efforts of Dr Steven Posniak, Dr Johnson Chung, Dr Xiao Liu, Dr DS Abdullah Al Maruf, Mr Kai Cheng, Prof Jonathan Clark, A/Prof Daniel Steffens, Prof Gordon Wallace and A/Prof Payal Mukherjee.

This study is under review for publication with Cell and Tissue Banking.

Chapter 4:

This conclusion chapter was researched and drafted by me in its entirety.

I acknowledge that an electronic copy of this thesis will be lodged for collection with the University of Sydney Library and that in-line with University of Sydney guidelines, this thesis does not exceed 60,000 words.

Dr Michael Fook-Ho Lee

16th December 2023

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

A/Prof Daniel Steffens

16th December 2023

A/Prof Payal Mukherjee

16th December 2023

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Abstract

Reconstruction of cartilaginous structures of the head and neck is a field that's been traditionally limited by difficulties sourcing sufficient donor cartilage, post-operative infection and achieving consistent aesthetic and functional results. Bioprinting is a technique that utilises 3D printed scaffolding with embedded bioinks typically containing living cells, supporting structures and hydrogel matrices to create customized implants that could address some of the limitations of contemporary reconstructive techniques.

The development and clinical translation of bioprinting into real word reconstructive surgery is an extensive ongoing task requiring the collaboration of scientists, engineers, and surgeons. In this thesis, we hope to better inform the choice of living cell types for cell-laden bioinks through answering two key questions:

1. Is there a role for co-culture of chondrocytes with stem cells as a method of expanding effective chondrocyte populations – helping to address limitations where sufficient donor cartilage is unavailable.
2. Are there any key differences between the harvest, digestion and proliferation of auricular and nasal septal chondrocytes – helping to better understand the impact of different donor sites on chondrocytes and its impact on clinical practice.

Our thesis concludes that co-culture of chondrocytes with stem cells at the same cell density is at least equivalent to mono-culture of chondrocytes, supporting its use as a technique for expanding effective chondrocyte populations and reducing the quantity of donor cartilage required. This thesis also found both auricular and nasal septal cartilage could be safely harvested from the concha and nasal septum respectively and kept free of contamination prior to digestion utilising an antibiotic solution, with no significant differences in proliferation rates between the two types.

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Chapter 1: Introduction

1.1 Reconstructive surgery of the head and neck

a. The role of cartilage in the head and neck

Cartilage is an important tissue of the head and neck, that serves as a key building block of multiple structures including the ear (pinna), nose (septum, alar and lateral cartilages) and larynx (epiglottis, corniculate, cuneiform, arytenoid, cricoid and thyroid cartilages). Additionally, cartilage is an important material used as a graft in many head and neck procedures including pinna reconstruction, tympanoplasty and rhinoplasty.

The absence of cartilage because of congenital defects such as microtia, or the loss of cartilage following trauma or ablative surgery, poses a complex challenge for reconstructive surgery.

b. Autologous reconstructive surgery

In the modern era, the use of autologous frameworks using tissue harvested from the same patient for ear and nasal reconstruction remains the gold standard despite also being among the earliest documented techniques, with methods for earlobe repair and rhinoplasty using local flaps first documented as early as 600 BCE in the Sushruta Samhita(1). In the Renaissance era, victims of war, syphilis and corporeal punishment often used metal facial prostheses in lieu of undergoing reconstructive surgery. In the 16th century, Gaspare Tagliacozzi, considered by many to be the founder of plastic surgery, would go on to detail the use of a delayed pedicled flap raised from the arm to reconstruct pinna, nasal and lip deformities(2).

Whilst the work of Sushruta and Tagliacozzi paved the way for modern autologous reconstructive techniques, significant groundwork did not occur until the 20th century. Non-cartilaginous sources such as calvarial and iliac crest bone saw mixed use in the late 19th century for nasal reconstruction, with Viktor Schmieden being among the first to recognise the value of autologous cartilage, using free cartilage grafts for auricular reconstruction in 1908 by harvesting costal cartilage and implanting this under abdominal skin to be later used as part of a delayed pedicled flap(3). Harold Gillies would go on to attempt total autologous cartilaginous reconstruction of the ear in 1920 through a hairline mastoid incision with the neocartilage subsequently separated from the head via a staged cervical flap, although by 1950, only 165 cases of total auricular reconstruction had been reported(4,5). Though initial cosmetic results

were not satisfactory, the use of costal cartilage has gone on to become a mainstay of autologous cartilaginous reconstructive techniques.

Radford Tanzer was the first to describe a reliable staged method of total auricular reconstruction for microtia with good cosmetic outcomes using autologous costal cartilage grafts in 1959, publishing a four-stage technique(6):

1. The existing lobular tissue is rotated to match a vertically mirrored pre-traced pattern of the contralateral ear
2. Time was allowed for induration to settle, following which, costal cartilage was harvested, stripped of periosteum and contoured to match a vertically mirrored plaster model of the contralateral ear then implanted within a subcutaneous pocket
3. The implant was allowed 4 months to consolidate, following which the implanted structure was manipulated to form a sulcus with a split thickness skin graft placed over any exposed cartilage
4. Following a further 6 weeks, a tragus and conchal floor was formed with the aid of a full thickness skin graft

This technique would be refined by Burt Brent, Tanzer's colleague, who went on to perform over 1,200 cases over his career and reduced the technique to a three-stage technique(7). Satoru Nagata would refine this further to a two-stage technique in 1993, with David Fisher finally proposing a single stage technique in 2014(8,9).

Currently, autologous reconstructive techniques using costal cartilage remain the gold standard for total auricular reconstruction in patients with microtia, though some significant limitations remain. Most notably is the significant learning curve involved, with aesthetic outcomes being highly variable and dependent on the surgeon's artistic ability in shaping the construct and technical skill in reducing operative failure due to soft-tissue necrosis(9–11). Additionally, whilst advancements in costal cartilage harvest have reduced donor site morbidity and the risk of chest wall deformation, the process of harvesting sufficient cartilage remains complicated by post-operative pain, keloid scarring, infection, pneumothorax, and wound dehiscence(10,12).

c. Homologous reconstructive surgery

Homologous allografts, transplanted tissue from a donor of the same species, have evolved as an alternative source of cartilage where autologous cartilage is insufficient or cannot be harvested due to technical difficulty or comorbidities. Building on his previous work with autologous cartilage, in 1937, Gillies trialling the use of maternal ears as a source of homologous cartilage for microtia reconstruction noted poor long-term results secondary to progressive resorption of the cartilage constructs across a series of over 30 ears(13,14). In 1955, Wallace Steffensen would utilise preserved costal cartilage and similarly to Gillies, would find the technique to be limited by significant resorption of the cartilaginous framework(15).

The use of irradiated cadaveric cartilage allografts was first described in 1961 by Reed Dingman, who utilised cadaveric costal cartilage harvested from donors at autopsy and preserved using cobalt radiation finding it to be a viable alternative to autologous sources for ear reconstruction(16).

Homologous cartilage has also seen use in other aspects of head and neck reconstruction, in particular facial augmentation. David Schuller utilised homologous costal cartilage for facial contour restoration on a series of 145 implants in a 1977 study and whilst initial results supported a low rate of complications at 36 months follow-up, longer term follow-up of these same patients by Duane Bradley Welling in 1988 found resorption rates of 75%.

Irradiated homologous costal cartilage has found particular use in rhinoplasty. This was first described by George Lefkovits in 1990, who utilised irradiated homologous costal cartilage in dorsal nasal augmentation finding advantages in avoiding the need for a donor site, low rates of resorption but potential complications were also noted including distortion or warping of the cartilaginous framework(17). In a direct comparison between irradiated homologous costal cartilage and autologous costal cartilage for rhinoplasty, like previous studies, Jee Hye Wee found higher rates of resorption with homologous cartilage. Additionally, autologous cartilage resulted in superior patient satisfaction scores and constructs more akin to native cartilage on histology(18). However, a subsequent 2021 meta-analysis by Nikita Kadakia found comparable rates of resorption, warping, infection, mobility and need for graft removal/replacement when compared with autologous costal cartilage grafts noting the previous findings to the contrary may have been limited by sample size (19).

In recent years, irradiated homologous costal cartilage has seen some early translational use in pinna reconstruction for microtia, ossicular reconstruction and laryngotracheoplasty(20–22). While some proposed limitations of the technique include the low cost-effectiveness and risk of disease transmission, studies have demonstrated a comparable cost profile with autologous costal cartilage and to date there are no reported cases of disease transmission resulting from irradiated homologous costal cartilage grafts in rhinoplasty despite its widespread use(23,24).

d. Alloplastic reconstructive surgery

Alloplastic frameworks, natural or synthetic materials not derived from a human or animal source, represent a third option that has historically been used for reconstructive surgery. Reports dating back to the 19th century detailed the use of various materials including gold, ivory, paraffin, silver wire, rubber and steel for reconstruction of the ear and nose(25,26). The primary proposed advantages of alloplastic materials were their relative abundance and the ability to avoid the donor site morbidity that cartilage harvest for autologous techniques entail. Despite his successes with autologous cartilage ear reconstruction, in a 1963 follow-up paper Tanzer proposed an ideal material would need to be inorganic and readily moulded preoperatively, sterilised, and buried subcutaneously(27).

Silicone rubber would be first proposed for ear reconstruction by Thomas Cronin in 1966 citing its biological inertness, softness and moldability as potential advantages(28). However, whilst highly aesthetic results were able to be produced, there was significant concern over the high degree of implant extrusion(29). One proposed reason for the failure of silicone implants was the inability of the inert implant to facilitate tissue ingrowth. Whilst subsequent techniques aimed to combat this with the inclusion of a porous covering mesh or the use of perforated silastic implants to allow tissue ingrowth, extrusion rates remained unsatisfactorily high(30).

Porous polyethylene is an alternative alloplastic material, first described as a viable option for soft tissue prosthesis of the nose and ears in 1946 by Leonard Rubin(31). Whilst early use of porous polyethylene saw that reasonable cosmetic results were achieved with low allergy, the material would be further popularised by Alexander Berghaus who noted its good rates of shape stability, tissue tolerance and low resorption especially when compared with other alloplastic materials for pinna reconstruction. In particular, the porous nature of polyethylene allowed for improved vascular ingrowth and collagen deposition with significantly lower rates of extrusion

as compared to silicone implants. However, Berghaus also noted unacceptable rates of implant rejection when porous polyethylene was used in the nasal septum(32).

The use of porous polyethylene for ear reconstruction was greatly popularised by John Reinisch who in 2009 described a technique using porous polyethylene and a two layered temporoparietal fascial/subgaleal flap that offered good aesthetic results and significantly reduced rates of implant extrusion and fracture compared to prior attempts(33). However, Reinisch would also note poorer flexibility and high risks of implant extrusion compared to autologous techniques when the covering flap was inadequate(34). To this day, the use of a covering temporoparietal flap remains a key step in ear reconstruction methods regardless of implant material used.

Despite this, while autologous reconstruction remains the gold standard, alloplastic reconstruction of the external ear using porous polyethylene is among the most used alternatives. A 2022 review by Yangmyung Ma noted the main advantages of alloplastic reconstruction to be more consistent aesthetic outcomes and bypassing of donor site morbidity, though despite being the most viable alloplastic option, porous polyethylene continued to have significantly higher rates of extrusion and infection compared to autologous techniques(35).

e. Challenges in ear reconstruction

When reviewing the history of cartilaginous reconstructive techniques, some of the biggest challenges in creating a complex 3D structure becomes apparent:

- Macroscopically, a graft needs to achieve a functional and aesthetic replication of the native structure's shape and this needs to occur in a consistent manner.
- Microscopically, the graft material needs to be biocompatible, bioactive and biodegradable.
- Logistically, the graft material needs to be economic to source with low or no donor site morbidity.

Whilst contemporary autologous, homologous and alloplastic techniques all have advantages in addressing some of these challenges, none of these techniques are able to reliably satisfy all criteria (Table 1).

Table 1: Comparison of reconstructive techniques for ear reconstruction

Reconstructive technique	**Macroscopic**	**Microscopic**	**Logistic**
Autologous	Highly durable implants with low infection rates Limited by experience and technical skill	Good biocompatibility, bioactivity, and biodegradability	Donor site risks (pain, scarring, infection, pneumothorax, wound dehiscence) Limited by available donor tissue May require multiple operations depending on method used
Homologous	Durable implants with low infection rates Limited by experience and technical skill	Good biocompatibility, bioactivity, and biodegradability Weak evidence with high resorption rates outside of rhinoplasty	Single stage operation No donor site morbidity
Alloplastic	More consistent aesthetic outcomes High rates of implant extrusion, infection, and fracture	Variable biocompatibility and biodegradability depending on material used No bioactivity	Single stage operation No donor site morbidity

When exploring how this applies to ear reconstruction in patients with microtia or following partial/total pinnectomy, autologous techniques must account for the limitations in harvesting cartilage in sufficient quantity, whilst also overcoming the challenges in forming a sufficiently

aesthetic result that is comparable to the contralateral ear which requires a great deal of surgical dexterity. Given the complex 3D structure of the ear, it is difficult for surgeons to consistently achieve functional and aesthetic results. Additionally, acquiring sufficient chondrocytes is a particular challenge for ears, where most of the cartilage is elastic cartilage, with conventional sources of chondrocyte harvest such as the rib or septum being comprised of hyaline cartilage and lacking the pliability and memory that is characteristic of elastic cartilage(36). As the cartilage type of different head and neck structures vary between elastic or hyaline, it is important to consider the lineage of donor chondrocytes (Table 2).

Table 2: Cartilage type of head and neck structures

Region	**Structure**	**Cartilage type**
Ear	Pinna	Elastic cartilage
	External auditory meatus	Elastic cartilage
	Eustachian tube	Elastic cartilage
Nose	Nasal septum	Hyaline cartilage
	Lateral nasal cartilage	Hyaline cartilage
	Nasal ala	Hyaline cartilage
Larynx	Epiglottis	Elastic cartilage
	Corniculate cartilage	Elastic cartilage
	Cuneiform cartilage	Elastic cartilage
	Arytenoid cartilage	Hyaline cartilage
	Cricoid cartilage	Hyaline cartilage
	Thyroid cartilage	Hyaline cartilage
	Tracheal rings	Hyaline cartilage

1.2 Advances in tissue engineering

a. Overview of tissue engineering

Tissue engineering represents an innovative solution that could overcome the limitations of existing cartilaginous reconstruction techniques of the head and neck. Techniques using tissue

engineering aim to combine living cells with scaffolds to create substitute tissue to replace damaged or diseased native tissue.

Several contemporary innovations have portended the viability of tissue engineering techniques. Computed tomography and magnetic resonance imaging, combined with computer simulation now allows for precise 3D modelling and when combined with 3D printers, allows for the creation of constructs with a high degree of fidelity. Interestingly, cartilaginous ear reconstruction was amongst the most publicised early proposed uses of tissue engineering. This was in part due to the work of Charles Vacanti, who's photos of a human ear shaped construct grown on the back of a mouse using bovine cartilage cells seeded onto a biodegradable polyglycolic acid scaffold were widely disseminated in the media in the 1990s(37).

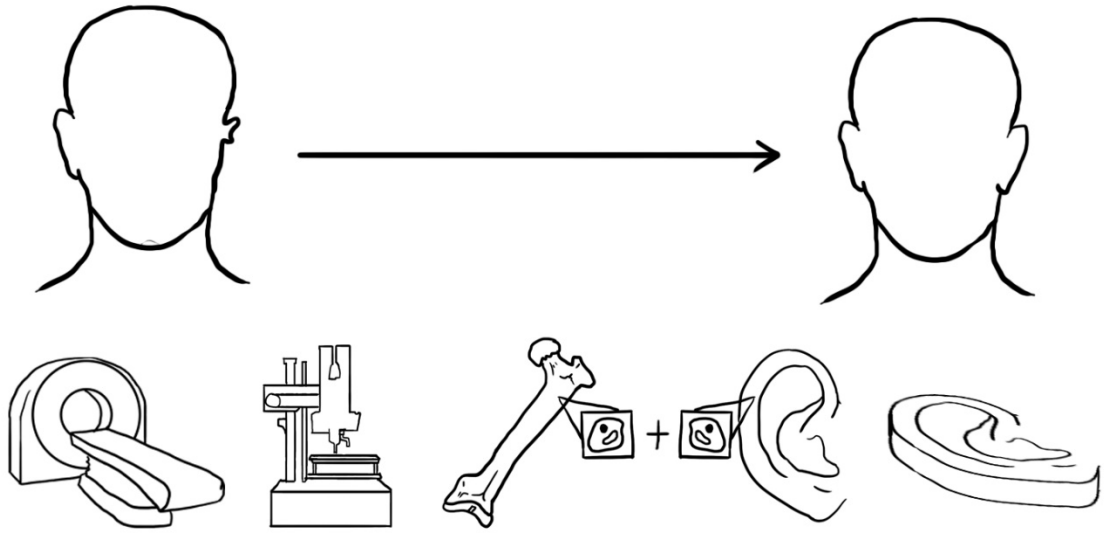
A scoping study by Weston Niermeyer in 2020 summarises the increasing interest of tissue engineering in head and neck applications over the past two decades, with tissue engineering techniques proposed for reconstruction of nasal, pinna, ossicular, craniofacial and laryngotracheal defects. However, it is noted studies remain largely at an *in vitro* or animal proof of concept stage, with a lack of large animal or human studies(38).

b. Bioprinters and bioinks

3D printing was first innovated in 1983 by Chuck Hull, then known as stereolithography, for use in the aerospace and automotive manufacturing industries(39). Through a method known as slicing, a 3D design can be represented as a series of 2D "slices", which in turn can be reproduced through layered printing allowing for the creation of precise 3D constructs. Among the earliest translations of this technology to the healthcare industry was in dentistry, for the creation of customised dental implants and prosthesis(40).

The utilisation of printing technology to deposit living cells, was a process dubbed cytoscribing, first proposed by Robert Klebe in 1986(41). Klebe's initial proposal and subsequent experiments used a custom modified commercial 2D inkjet printer to deposit cell adhesion proteins and monoclonal antibodies onto a substrate.

Figure 1: 3D bioprinting process to form an ear shaped construct. CT/MRI imaging of contralateral ear, 3D bioprinter, stem cells harvested from bone marrow, chondrocytes harvested from auricular cartilage, 3D bioprinted pinna construct (left to right).



The combination of 3D printers with cytoscribing, for 3D bioprinting as part of reconstructive surgery, requires 3 stages: pre-processing, processing, and post-processing. In microtia reconstruction, pre-processing refers to modelling of the 3D construct by vertically mirroring computed tomography/magnetic resonance imaging of the contralateral ear to form a symmetrical ear model. In the processing stage, living cells are proliferated and combined with one or more biomaterials, termed bioinks, and deposited using a 3D printer to form a 3D ear shaped construct (Figure 1). Before transplantation, in the post-processing stage the construct is placed in a bioreactor to maintain its biological and mechanical properties(42).

Subsequent printers have been designed specifically for use in tissue engineering and can be broadly divided into three classes:

- Inkjet printing, wherein bioinks are deposited through a nozzle by pneumatic pressure in the form of droplets.
- Extrusion printing, wherein bioinks are heated and extruded through a nozzle as a linear structure which subsequently solidifies.
- Laser printing, wherein bioink droplets are deposited using a pulsed laser beam through the process of laser-induced forward transfer.

Several printers operating on these variable modalities have been developed, with their respective advantages and disadvantages (Table 3)(42,43).

Table 3: Advantages and disadvantages of bioprinting technique modalities

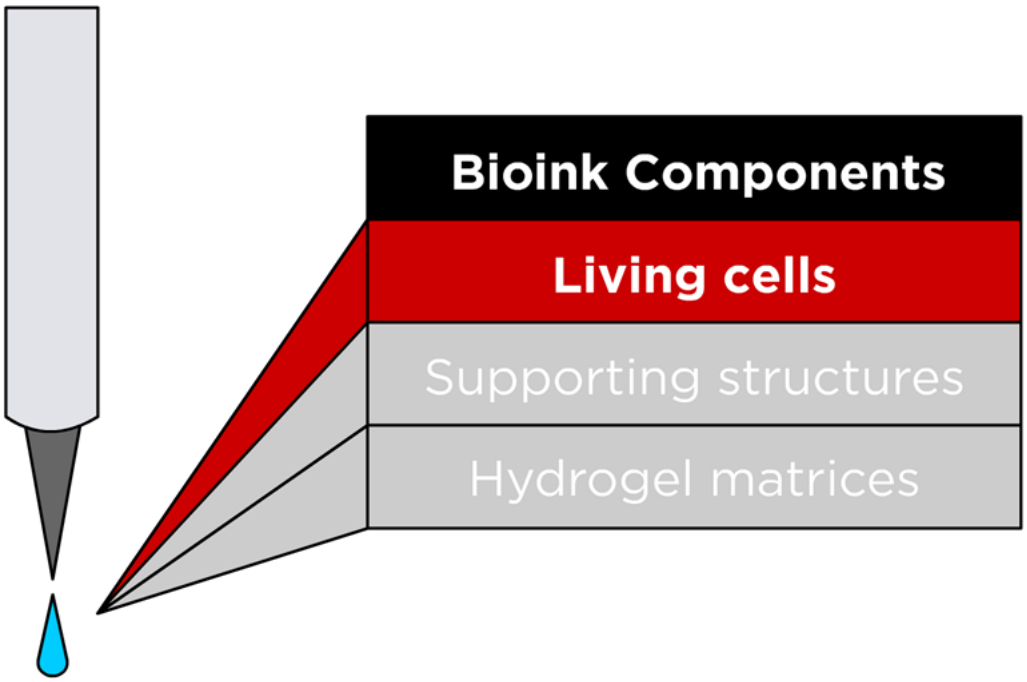
Bioprinting technique	**Advantages**	**Disadvantages**
Inkjet	High spatial resolution Contactless printing	High viscosity bioinks prone to clogging
Extrusion	High structural integrity	Lower spatial resolution Stress induced cell deformation Limited material options
Laser	Highest spatial resolution No risk of nozzle clogging	Lower cell viability due to cell death from laser energy

Whilst the use of hydrogels to support cell proliferation and differentiation in bioinks is effective and seeing use in many conventional bioprinting techniques, the poor structural integrity and mechanical properties of many hydrogels precludes their use in creating complex structures such as the human ear. Hybrid printing represents a recent innovation wherein cell-laden hydrogels are printed in tandem with structural supports, in a technique that seeks to provide improved mechanical integrity without compromising cell viability.(44).

c. Bioink composition for ear reconstruction

Bioinks typically consists of three components: living cells, supporting structures and hydrogel matrices (Figure 2). Like bioprinting modalities, there are several cell types and materials seeing use in bioinks. It has been proposed that an ideal bioink should have similar mechanical, rheological and biological properties of the target tissue being replicated(45).

Figure 2: Primary components to bioinks for bioprinting and tissue engineering



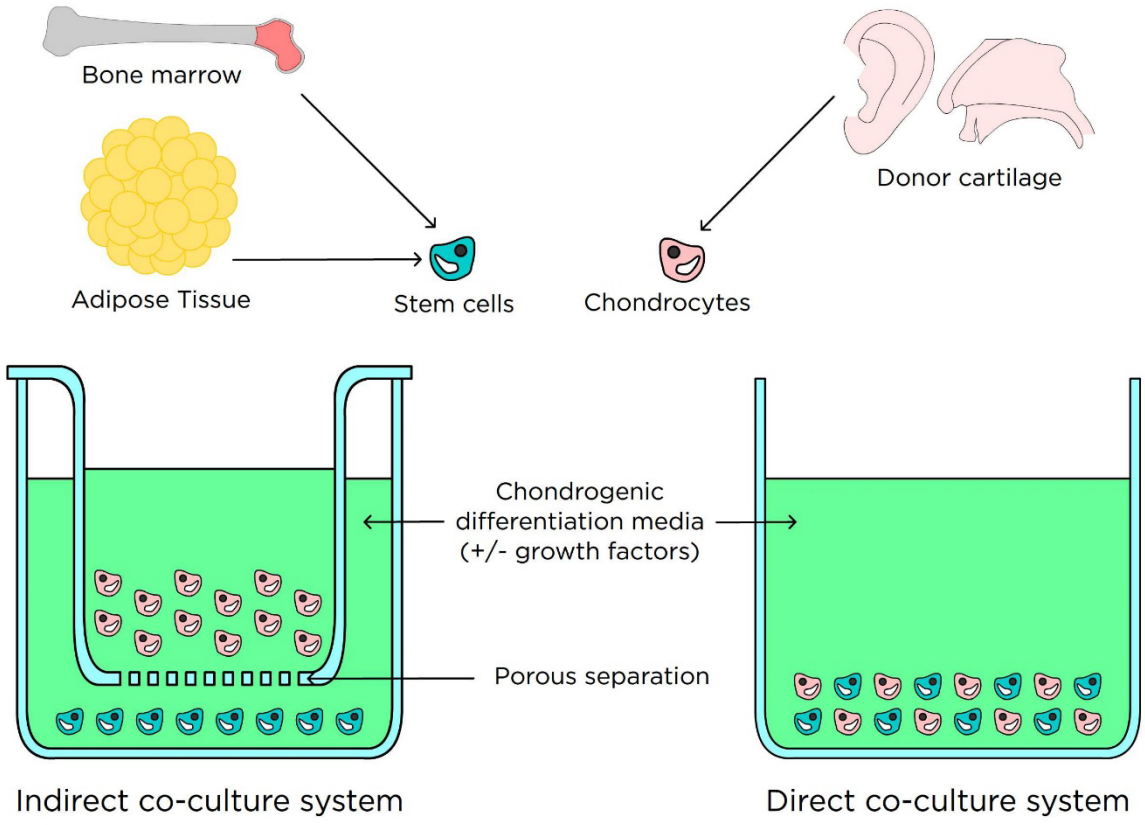
Native ear cartilage is comprised of elastic cartilage and has numerous constituents including elastin, collagen (type II, IX, X, XI), glycosaminoglycan, aggrecan and biglycan that provide the ear its elastic and compressive properties(46). While native auricular chondrocytes would be the natural choice as the living cell line to mimic these properties, being terminally differentiated cells, they have a limited capacity for self-renewal meaning constructs are absorbed rather than maintained over time(47). Additionally, there is a significant limitation on the amount of native auricular cartilage available for chondrocyte harvest. Chondrocyte populations cannot be extensively expanded alone in culture, as this results in the formation of fibrous, mechanically inferior constructs(48).

Comparatively, stem cells are relatively abundant from a variety of autologous tissue sources, possess the ability for self-renewal and chondrogenic differentiation can be induced by growth factors under the right conditions(47,49). However, despite these advantages, constructs using stem cells alone are limited by high rates of mineralisation and ossification *in vivo*(49).

Co-culture has emerged as a powerful method of capitalising on the properties of both chondrocyte and stem cells. When cultured in the same environment, chondrocytes can induce chondrogenic differentiation in stem cells with reduced incidence of hypertrophy and tissue calcification compared to culture systems of stem cells and growth factors alone (Figure 3)(50).

Additionally, there is evidence to suggest co-culture systems exhibit increased chondrogenic activity compared to mono-culture of chondrocytes, reducing the number of chondrocytes required and potentially overcoming the challenge of having limited native donor cartilage available for autologous chondrocyte harvest(51,52).

Figure 3: Components of chondrocyte/stem cell co-culture systems. Stem cells harvested from bone marrow or adipose tissue sources. Chondrocytes harvested from autologous cartilage sources (ear and nasal septum pictured). Left: Indirect co-sculture using a Transwell system. Right: Direct co-culture.



Whilst the use of co-culture for cartilage regeneration is seeing increasing use in preliminary studies to facilitate cartilage repair in orthopaedic surgery for treatment of osteoarthritis, there remains limited studies investigating the use of co-culture for head and neck reconstruction and utilising chondrocytes sourced from the head and neck(53–55).

1.3 Aims and structure of thesis

The goal of this thesis is to synthesise the current reported co-culture techniques relevant for the head and neck and to investigate the behavior of different donor sites for chondrocytes and

its impact on clinical practice. Chapter 2 of the thesis is a systematic review of studies detailing co-culture utilising chondrocytes derived from and/or for use in the head and neck. Chapter 3 of the thesis details an animal study wherein sheep auricular and nasal septal cartilage was harvested, digested, and proliferated with chondrocyte counts between the two groups compared. Chapter 4 of the thesis summarises these findings and proposes future directions of investigation for co-culture in head and neck reconstruction.

Chapter 2: Systematic review - Co-culture of chondrocytes and stem cells: a review of head and neck sourced chondrocytes and stem cells for cartilage regeneration

2.1 Abstract

Introduction: Bioprinting, using "bioinks" consisting of living cells, supporting structures and biological motifs to create customized constructs, is an emerging technique that aims to overcome the challenges of cartilaginous reconstruction of head and neck structures. Several living cell types and culturing methods have been explored as bioinks with varying efficacy. Co-culture of primary chondrocytes and stem cells (SCs) is one technique, well established for degenerative joint disease treatment, with potential for use in expanding chondrocyte populations for bioinks.

This study aims to evaluate the techniques for co-culture of primary chondrocytes and MSCs and their outcomes for head and neck cartilage regeneration.

Methods: A literature review was performed through OVID/Web of Science/MEDLINE/BIOSIS Previews/Embase. Studies reporting on chondrocytes and SCs in conjunction with co-culture or cartilage regeneration were included. Studies not reporting on findings from chondrocytes/SCs of the head and neck were excluded. Extracted data included cell sources, co-culture ratios and histological, biochemical and clinical outcomes.

Results: 15 studies met inclusion criteria. Auricular cartilage was the most common chondrocyte source (n=10), then nasal septum (n=5), articular (n=1) and tracheal cartilage (n=1). Bone marrow was the most common SC source (n=9) then adipose tissue (n=7). Techniques varied, with co-culture ratios ranging from 1:1 to 1:10. All studies reported co-culture to be superior to SC mono-culture by all outcomes. Most studies reported superiority or equivalence of co-culture to chondrocyte mono-culture by all outcomes. When comparing clinical outcomes, co-culture constructs were equivalent to chondrocyte mono-culture in diameter, and equivalent or inferior in wet weight and height.

Conclusion: Co-culture of primary chondrocytes and SCs is a promising technique for expanding chondrocyte populations, with at least equivalence to chondrocyte mono-culture and superior to SC mono-culture when seeded at the same chondrocyte densities. However, there remains a lack of consensus regarding the optimal cell sources and co-culture ratios.

Keywords: Bioprinting, reconstructive surgery, chondrocytes, stem cells, co-culture

2.2 Introduction

Cartilage is a ubiquitous tissue in the head and neck, being present in the pinna, nasal septum and alar, larynx and trachea. Furthermore, it is a commonly used graft material for several procedures including rhinoplasty, myringoplasty and laryngotracheal reconstruction. Due to the limited ability for cartilage to self-regenerate and repair, congenital and acquired cartilaginous abnormalities of the head and neck remain complex reconstructive challenges.

The current gold standard is the use of autologous tissue, such as native costal cartilage(56). However, this comes at the expense of donor site morbidity and due to the complexity of cartilaginous structures in the head and neck, achieving optimal results require a high degree of surgical skill(57–59).

For conditions such as microtia, the use of alloplastic materials reduces donor site morbidity and allows for additive manufacturing techniques, deposition of materials in structured layers according to computer-aided designs, to replicate complex 3 Dimensional (3D) anatomy. However, this method is associated with risks of implant extrusion and infection(60).

An emerging reconstructive technique that seeks to address these limitations is the use of bioprinting to create customized 3D constructs for implantation. These constructs can serve as a scaffold for tissue engineered “bioinks,” to create implants that can be integrated with human tissue in a way which is both functional and aesthetic(44). Bioinks are printable biomaterials, with biocompatibility and biodegradability. For 3D printing, cell-laden bioinks typically consist of three components: living cells, supporting structures such as hydrogel matrices and additional biological motifs such as growth factors(61).

Several cell culturing methods utilizing chondrocytes, stem cells or a combination of both have been proposed for the living cell component of the “bioink” with the aim of optimizing growth, cell differentiation and maintenance of the phenotype. Harvesting chondrocytes in sufficient quantities poses a challenge and when used alone, their limited capacity for self-renewal leads to formation of fibrous, mechanically inferior constructs as compared to native cartilage(62). From a practical standpoint, it is also necessary to preferentially consider chondrocytes from a local head and neck donor site, to reduce the morbidity from creating a secondary donor site. Comparatively, while stem cells (SCs) can be harvested in larger numbers, when used alone results in high rates of mineralization and ossification *in vivo*(49). One technique that seeks to

overcome the limitations of these two cell types is co-culture, wherein harvested chondrocytes are cultured in conjunction with stem cells creating a synergistic effect that allows for maintenance of the chondrocyte phenotype. When cultured together, the pluripotent nature of SCs allows for similar quantities of cartilage to be produced as cultures of chondrocytes alone at the same cell density(63,64).

While co-culture as a technique is established for the treatment of degenerative joint disease, the majority of existing literature focuses on the use of articular cartilage a paucity of studies exploring the use of co-culture for expansion of chondrocytes harvested from the head and neck in local reconstructive surgery. This systematic review aims to better inform the creation of bioinks in exploring one constituent component, living cell type, through comparing techniques for co-culture of primary chondrocytes derived from the head and neck and stem cells. and their histological, biochemical and clinical chondrogenic outcomes as compared to mono-culture. The other constituent components of bioinks, supporting structures and biological motifs, fall outside the scope of this review.

2.3 Methods

A systematic literature review was conducted to identify studies exploring the use of primary chondrocytes of the head and neck and SCs in co-culture. A written protocol was developed in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines and registered with the Center for Open Science (OSF) (DOI 10.17605/OSF.IO/UM95Y)(65,66).

a. Search strategy

A systematic electronic search was conducted through MEDLINE (1946 –), Embase (1947 –) via Ovid and BIOSIS Previews (1926 –) on May 21st 2021 assisted by an experienced librarian from the University of Sydney. A search strategy was designed for each database to identify all relevant studies on co-culture. A combination of search terms including “co-culture,” “cartilage regeneration,” “tissue regeneration,” “stem cells,” and “mesenchymal stromal cells”. The search strategy was adopted to each individual database to optimise search coverage. The targets were case reports, case series, *in vitro* and *in vivo* studies reporting on the use of chondrocytes with SCs in co-culture for tissue engineering.

b. Study selection

The primary reviewer (ML) independently assessed the titles and abstracts of the search results. Irrelevant studies were disregarded whilst those deemed to be relevant were assessed using a structured data collection form. Full texts of relevant articles were then thoroughly examined for compliance with the eligibility criteria to determine study inclusion. The primary reason for study exclusion was noted.

c. Inclusion and exclusion criteria

All articles that were written in or translated to English, published in a peer-reviewed journal and specifically discussed the use of chondrocytes and stem cells in co-culture and their outcomes for chondrogenesis in the head and neck were considered. Articles reviewing mono-culture techniques for chondrocytes or stem cells alone were excluded. Articles discussing the use of chondrocytes harvested from sources outside the head and neck were excluded unless specifically investigating their use in head and neck reconstructive surgery. A citation search was performed on included articles and cross referenced. For included studies, data was collected on chondrocyte and stem cell sources, study duration, co-culture ratios used and histological (extracellular matrix (ECM) deposition, elastin fibres, lacuna structure), biochemical (aggrecan/biglycan, glycosaminoglycan, COL2A1, SOX9, ACAN) and clinical (wet weight, diameter, height) as summarized in Table 1. The QUIPS tool was used to assess for risk of bias in all studies(67).

2.4 Results

The search strategy identified 331 articles of which 164 were removed as duplicates. After screening and application of inclusion and exclusion criteria, 30 articles were identified. An additional 5 studies were reviewed following screening of citations. Further screening of these 35 full text articles yielded 15 studies that were included in this review (shown in Fig. 1)(52,68–81).

Studies compared the relative chondrogenic potential between co-culture and mono-culture groups using 11 different qualitative and quantitative measures. The control group for these studies consisted of mono-culture of chondrocytes and/or mono-culture of SCs with an

equivalent total cell count to the co-culture groups. Chondrogenic potential was determined by deposition of extracellular matrix (ECM) or components including aggrecan/biglycan, glycosaminoglycan, elastin/elastic fibres, extracellular matrix (ECM) deposition and lacuna structures identified under microscopy with staining as evidence of chondrogenesis, as well as assays for surrogate chondrogenic gene markers including COL2A1, SOX9 and ACAN. Wet weight, diameter and height were also measured to offer a quantitative comparator.

a. Assessment of heterogeneity of published literature

Six studies came from China (71,74–77,80), three from the United States (72,79,81), two from Sweden (70,78), two from Switzerland (52,68) and one from Canada (69) and Malaysia (73), respectively. Six studies reported on *in vitro* findings alone, five on *in vivo* findings and four on *in vitro* and *in vivo* findings. The quality of these studies is summarized in Table 2.

b. Cell source

Auricular cartilage was the most common source of primary chondrocytes (n=10) followed by nasal septal cartilage (n=5), articular cartilage (n=2) and tracheal cartilage (n=1) with three studies reporting on findings utilizing two different sources. Bone marrow was the most common source of SCs (n=9) followed by adipose tissue (n=7) with one study reporting on findings using two different sources. In four studies, SC mono-culture was supplemented by the addition of transforming and/or insulin growth factor.

c. Maximal duration

Reported study lengths varied from 3 weeks to 3 months. In order to quantitatively compare endpoints, results at the maximal duration of each study were chosen.

d. Co-culture system

Studies reported on both indirect co-culture systems. Twelves studies reported on the use of direct co-culture, either suspended together in monolayer within a chondrogenic differentiation media or hydrogel, through formation of centrifuged pellets containing both cell line, or seeded together into printed constructs(52,69–81). Four studies reported on cell co-culture within subcutaneously implanted hydrogels, with Cai *et al.*, Morrison *et al.* and Pleumeekers *et al.* utilizing Pluronic F-127, type I collagen, sodium alginate and type I collagen/hyaluronic acid hydrogels respectively(52,71,72,79). Two studies reported on cell co-culture within hydrogels

as part of bio-printed constructs, with Apelgren et al. and Moller et al. both using extrusion printed cell-laden Nanofibrillated Cellulose/Alginate (NFC/A) bioinks within scaffolding(70,78). Only one study reported on indirect co-culture, using a Transwell culture system(68).

e. Ratio

There was considerable variability in the reported chondrocyte to SC co-culture ratios utilized between the different studies. On the lower end, one study explored co-culture using a 1:10 ratio, with the highest chondrocyte ratio being 1:1 utilized in four studies. Other ratios explored from highest to lowest included 2:3 (n=1), 1:2 (n=1), 3:7 (n=3), 1:3 (n=3), 1:4 (n=4), 1:5 (n=3) and 1:9 (n=3). One study did not specify the co-culture ratio used and four studies compared findings between two or more different co-culture ratios.

f. ECM deposition

Zieger *et al*. was the only study that qualitatively assessed for ECM deposition under light microscopy with hematoxylin and eosin staining. When comparing co-culture of porcine auricular cartilage with human adipose tissue derived stem cells at 10 weeks, co-cultured groups exhibited more compact organization, suggestive of cartilage formation, when utilizing co-culture ratios of 1:9 as compared to ratios of 1:5 or mono-culture of stem cells(81).

Thirteen studies assessed for ECM deposition via the presence of ECM components or surrogate markers. Aggrecan/biglycan was assessed by expression of mRNA using PCR, with Goh *et al*. finding in a study on human auricular cartilage with human adipose derived stem cells that co-culture at an unspecified ratio supplemented with growth factor demonstrated increased aggrecan mRNA expression at 3 weeks compared to mono-culture of both auricular chondrocytes and adipose derived stem cells at the same cell density(73). Cai *et al*. found in a study on auricular chondrocytes with human adipose derived stem cells that co-culture at a ratio of 3:7 demonstrated equivalent aggrecan mRNA expression at 8 weeks to mono-culture of auricular chondrocytes at the same cell density (5.0×10^7), and higher aggrecan mRNA expression than mono-culture of adipose derived stem cells or auricular chondrocytes at a lower cell density (1.5×10^7)(71). Kang *et al*. similarly found porcine auricular cartilage co-cultured with porcine bone marrow derived stem cells at co-culture ratios of 1:1 demonstrated equivalent biglycan mRNA expression at 14 weeks to mono-culture of auricular chondrocytes,

and superior to mono-culture of bone marrow derived stem-cells and co-culture with a ratio of 1:9 respectively(75). All three studies demonstrated increased aggrecan/biglycan mRNA expression in co-culture groups when compared with mono-culture of SCs(71,73,75).

GAG deposition was the most assessed measure of ECM deposition, with two studies finding increased GAG deposition in co-culture compared to chondrocyte mono-culture groups(68,78). Acharya *et al*. found co-culture ratios of 1:3 results in increased GAG deposition compared to 1:9, and samples utilizing stem cells derived from human bone marrow to be superior to adipose tissue(68). Most of the studies, six of eleven, found GAG deposition to be similar in co-culture and chondrocyte mono-culture groups(52,71,72,76,79,80). Pleumeekers *et al*. found that whilst co-culture and chondrocyte mono-culture utilizing the same chondrocyte source resulted in similar GAG deposition, samples utilizing bovine nasal cartilage, both co-culture and chondrocyte mono-culture, were superior to samples utilizing auricular cartilage(52). Three studies found GAG deposition to be increased in chondrocyte mono-culture compared to co-culture groups(69,70,74). Kang *et al*. found whilst mono-culture of porcine articular chondrocytes demonstrated increased GAG deposition than all co-culture groups, co-culture ratios of 1:1, 2:3 and 3:7 were all similar and superior to lower ratios of 1:4 and 1:9(74). All eleven studies measuring GAG deposition found co-culture groups to be superior to SC mono-culture groups.

g. Chondrogenic gene expression

Twelve studies compared chondrogenic gene expression between co-culture and mono-culture groups using quantitative polymerase chain reaction (PCR) of type II collagen (COL2A1), SOX9 and aggrecan (ACAN). COL2A1 was the most assessed, with four studies finding increased COL2A1 expression in co-culture compared to chondrocyte mono-culture groups(73,77,79,81). Morrison *et al*. found co-culture ratios of 1:1, 1:2, 1:5 and 1:10 all demonstrated similar COL2A1 gene expression and increased compared to the chondrocyte mono-culture group. It is worth noting the study utilized both porcine auricular and tracheal chondrocytes, but no comparison was made between outcomes using these two chondrocyte sources(79). Ziegler *et al*. found co-culture ratios of 1:9 resulted in increased COL2A1 gene expression compared to ratios of 1:5 which were equivalent to chondrocyte mono-culture(81). Most of the studies, five of twelve, found COL2A1 gene expression to be similar in co-culture and chondrocyte mono-culture groups(71,74,76,79,80). Kang *et al*. found that co-culture ratios

of 1:1, 2:3 and 3:7 were all similar to mono-culture of porcine articular chondrocytes and superior to lower ratios of 1:4 and 1:9(74). Pleumeekers *et al*. found that whilst co-culture and chondrocyte mono-culture utilizing the same chondrocyte source resulted in similar COL2A1 gene expression, samples utilizing bovine nasal cartilage, both co-culture and chondrocyte mono-culture, were superior to samples utilizing auricular cartilage(52). Two studies found COL2A1 gene expression to be increased in chondrocyte mono-culture compared to co-culture groups(69,75). Kang *et al*. found mono-culture of porcine auricular cartilage to be superior to co-culture at ratios of 1:1, and both groups to be superior to co-culture of ratios of 1:9. All twelve studies measuring COL2A1 gene expression found co-culture at the optimal ratio described in each paper to be superior to SC mono-culture groups.

h. Weight/volume

Five studies compared the weight or volumetric measurements of the final co-culture or mono-culture constructs. None of the five studies found co-culture groups at the same cell density resulted in a construct of increased wet weight, diameter or height compared to chondrocyte mono-culture(69,71,72,74,80). Two studies found co-culture constructs to be of similar weight to chondrocyte mono-culture constructs, whilst two others found co-culture constructs to be of lower average weight(69,71,74,80). All three studies which compared diameters found co-culture constructs to be similar to chondrocyte mono-culture(72,74,80). Two studies compared height, with one finding co-culture constructs to be similar to chondrocyte mono-culture, and one finding co-culture constructs to be of lower average height(72,74). Kang *et al*. found mono-culture of porcine articular cartilage to be equivalent to co-culture of ratios of 1:1, 2:3, 3:7 and 1:4 when comparing the diameter of constructs at 8 weeks, and superior to co-culture at all ratios when comparing wet weight and height(74). All five studies found constructs in the co-culture groups to be superior to SC mono-culture groups when comparing wet weight, diameter and height.

i. Risk of bias

QUIPS assessment was performed on all studies, with one study at high risk of bias (78), seven at moderate risk of bias (68,73,74,77,79–81) and seven at low risk of bias (52,69–72,75,76). With all studies being pre-clinic in-vitro or in-vivo case control studies, there was no risk of

bias across all studies from either participation, attrition or confounding. Poor reporting on methods in several studies lead to moderate risk of bias in prognostic factor measurement in three studies and outcome measurement in two studies. Studies which clearly delineated the protocols used for cell culture and processing, as well as measuring outcomes were assessed as being low risk of bias and better inform future attempts at replicating their findings. Statistical methods used were inconsistent across five studies, with one study failing to address methods of statistical reporting and analysis entirely and at high risk of bias. Graphical representation of risk of bias findings was created using the *robvis* tool (shown in Fig. 2).

2.5 Discussion

Two cell types are seeing increasing use in tissue engineering for cartilaginous reconstructive surgery of the head and neck, primary chondrocytes and SCs (52,70–74,78–81). The use of co-culture techniques to expand chondrocyte populations is already being explored in pre-clinical orthopedic surgery trials where treatment of osteoarthritis, a degenerative disease of joint cartilage, can be augmented by local introduction of MSCs(53,54).

The use of primary chondrocytes as a in cell-laden bioinks for bioprinting is already an established technique(44,82). Traditional bioprinting techniques introduce chondrocytes and stem cells into a scaffold using a non-uniform manual process of cell seeding(83). The development of hybrid printing has allowed for uniform deposition of cells between layers of polymer scaffolding to support cell proliferation and stem cell differentiation(84). However, in order to replicate or fill a large cartilaginous structure or defect, a very large number of cells are required. Inevitably this faces the same disadvantages as autologous reconstruction, being the difficulty and morbidity involved in acquiring chondrocytes in sufficient quantities. When utilized alone, chondrocytes tend to change phenotype and de-differentiate to a fibroblastic lineage over numerous cell cycles preventing them from achieving sufficient expansion whilst generating an appropriate matrix that resembles native cartilage.

SCs can differentiate into many human tissues including cartilage under the appropriate conditions, such as exposure to transforming growth factors and bone morphogenic proteins.

They can be harvested in higher numbers from less morbid sources such as bone marrow and adipose tissue. They also have significantly higher proliferation ratees and are able to undergo multiple cycles of duplication without depreciation of their chondrogenic potential. However, when utilized alone, formed matrices tend to be unstable and more prone to calcification. This was demonstrated by Zhang *et al*. who noted that constructs consisting of SCs alone were shrunken with hypertrophic cartilaginous and fibrous tissue when compared with the chondrocyte mono-culture and co-culture groups(80).

The use of co-culture techniques seeks to overcome the limitations of both these cell sources, wherein chondrocytes and SCs are synergistically utilized together, with SCs able to maintain the chondrocyte phenotype allowing for increased proliferation and reduced de-differentiation even in the absence of growth factors(73). When co-cultured, the presence of SCs has been shown to increase the chondrogenic potential of chondrocytes and decrease the quantity of functional chondrocytes required. Comparison between co-culture and mono-culture subgroups is summarized in Table 3 (52,68–81). Co-culture was demonstrated to be a superior technique to mono-culture of MSCs across all studies. When comparing co-culture with mono-culture of chondrocytes, the results were more heterogeneous, with the majority of studies showing similar levels of chondrogenesis between the two groups. It is worth noting that comparator groups used the same total cell counts, so despite the similar chondrogenic potential noted in these studies, this was achieved in the co-culture group despite a lower volume of chondrocytes required. This is clinically relevant, as reducing the effective chondrocyte count required to repair a defect of a given size means surgeons will not be required to harvest as much donor cartilage thereby reducing donor site morbidity. When comparing wet weight and volumetric measures of cartilage formed, all studies demonstrated that co-culture was either equivalent or inferior to mono-culture of chondrocytes.

Co-culture systems can either be direct wherein physical contact between cells is facilitated, or indirect wherein cells are separated by a physical barrier such as a semi-permeable membrane which only enables paracrine signaling between the two cell types via soluble factors and cell-derived membranous structures(85). The efficacy of direct and indirect co-culture systems remains unclear, with juxtacrine signaling between adjacent cells and physical cell-cell contact reported as a key factor for chondrogenesis in direct co-culture systems, whilst Levorson *et al.*

found indirect co-culture systems resulted in increased GAG, collagen and ECM production(86,87). This was further supported by Pleumeekers *et al.* whose findings suggest paracrine signaling to be more important given their successful use of co-culture with alginate hydrogel despite alginate hindering direct cell-cell contact(52). Unfortunately, comparison between direct and indirect co-culture in this review was limited, as only one study explored indirect co-culture, using a Transwell system, with their finding that co-culture resulted in increased GAG expression than mono-culture of chondrocytes and SCs respectively being in line with the overall findings(68). The co-culture technique can be utilized to enhance the mechanical characteristics and chemical makeup of tissue-engineered cartilage. Additionally, it can be combined with other methods such as gene therapy or the delivery of growth factors to promote the differentiation and maturity of the cartilage tissue.

In translation to clinical applications in 3D constructs, the use of bioinks consisting of living cells, supporting structures and hydrogel matrices for 3D printing is required. Six studies reported on the use of hydrogels using a range of different biocompatible materials with the other nine studies either being unspecified or using chondrogenic differentiation media alone. Whilst the lack of standardization and reporting on the use and type of hydrogels prevented comparison between groups and is a limitation of this review, as all factors aside from cell line were standardized within each study, we were still able to draw direct comparisons between co-culture and mono-culture groups when examining studies individually. Three studies explored direct subcutaneous implantation of cell-laden hydrogels, all finding equivalent GAG expression between co-culture and chondrocyte mono-culture implants(52,71,79). By encapsulating cells within a high-density collagen hydrogel to form a pinna construct using a mold, Cohen *et al.* highlighted the potential for hydrogels to allow for constructs to go directly from generation to implantation without the need for lengthy culture, with the constructs formed by co-culture exhibiting equivalent COL2A1 expression, diameter and height when compared with those formed by chondrocyte mono-culture(72). Two studies explored 3D printing, both using cell-laden NFC/A bioinks within scaffolding(70,78). Moller *et al.* found one of the shortcomings of traditional hydrogels is their viscoelastic nature which limits printing resolution, finding the use of NFC/A bioinks overcame this limitation(78). Apelgren *et al.* similarly found NFC/A overcame this limitation and noted gelatine stabilized hydrogels may offer similar advantages(70). Co-culture techniques for living cells in bioinks has already

seen some early translational use with Posniak *et al.* reporting on the use of human septal chondrocytes co-cultured with bone marrow derived SCs extrusion printed in gelatine methacryloyl/methacrylated hyaluronic acid hydrogels between polycaprolactone scaffolding, finding co-culture yielded similar degrees of cell proliferation and chondrogenic expression to mono-culture of chondrocytes(88).

There were high levels of scientific vigor with low to moderate risk of bias across the majority of included studies. These pre-clinical findings form a strong basis for ongoing human trials into the use of chondrocyte co-culture techniques.

a. Chondrocyte source

The included studies harvested cartilage from auricular, nasal septal, articular, and tracheal cartilage sources. It is important to note that the cell source is relevant to its translation towards clinical use. Of these four cell sources, only auricular cartilage is composed of elastic cartilage, whilst nasal septal, articular and tracheal cartilage are all composed of hyaline cartilage without elastic fibres potentially limiting their use for reconstruction of the ear. Additionally, harvesting of tracheal cartilage would likely be contraindicated due to significant donor site morbidity and is therefore not clinically translatable.

When comparing studies using auricular chondrocytes alone, findings were similar with co-culture yielding greater or equal chondrogenic potential compared to mono-culture of auricular chondrocytes or SCs in the majority of studies (shown in Table 4)(71–73,75–77,80,81). The only outlier was Kang *et al*. which found mono-culture of auricular chondrocytes to be associated with increased COL2A1 expression compared to co-culture, though co-culture at ratios of 1:1 was equivalent or superior across other measures of chondrogenic potential including biglycan, lacuna structures, elastic fibres, SOX9 and ACAN(75).

Pleumeekers *et al*. was the only study which compared chondrogenic potential between two different chondrocyte sources, findings that both co-culture and mono-culture of nasal septal chondrocytes were associated with higher expression of GAG and COL2A1 compared to auricular chondrocyte groups. There was no significant difference between the co-culture and

mono-culture groups of the respective cell types, and both groups were superior to mono-culture of SCs(52).

b. Co-culture ratio

The most efficient mixture of remains debated in reported literature, with Apelgren *et al.* noting the most efficient reported mixing ratio of chondrocytes and SCs being 1:4(70). This is reflected in our findings, with the most utilized ratio between studies being 1:4. In a 2014 concise review of SC co-cultures, de Windt *et al.* found the optimal ratio for chondrogenesis in published studies that directly compared two or more ratios to vary from 1:1 up to 1:9(89).

As the co-culture protocols varied between studies, direct comparison of outcomes between studies utilizing different co-culture ratios was not possible. This is highlighted when comparing Anderson-Baron *et al.*'s finding that chondrocyte and SC co-cultures at ratios of 1:3 demonstrated inferior GAG expression to mono-culture of chondrocytes, with Acharya *et al.* who found co-culture to be superior to mono-culture of chondrocytes at the same 1:3 ratio, however using a different study protocol(68,69). For the four studies comparing different ratios for co-culture with all other factors kept standardized, two studies showed the chondrogenic potential when using a chondrocyte to SC co-culture ratio of 1:1 was superior to higher SC ratios up to 1:9 with ratios of 1:9 resulting in no cartilaginous tissue formation, one study showed using a ratio of 1:1 was similar to ratios up to 1:10 and another conversely showed a ratio of 1:9 was superior to 1:5(74,75,79,81).Whilst the ideal co-culture ratio as reported in the literature remains unclear, in approaching clinical translation there would be a benefit to identifying the highest acceptable ratio, as this would require a lower amount of primary chondrocytes to be harvested. Future studies directly comparing cell counts using different ratios, replicating similar protocols to existing studies that support the efficacy of higher co-culture ratios, could better inform optimal co-culture ratios for clinical use.

c. Stem cell source

All studies sourced stem cells either from bone marrow (bone marrow derived stem cells) or adipose tissue (adipose tissue derived stem cells). Acharya *et al*. was the only study to compare between the two groups, reporting higher glycosaminoglycan expression in the co-culture when

using SCs sourced from bone marrow as opposed to adipose tissue(68). This is supported in the literature which notes bone marrow stem cells tend to exhibit higher chondrogenic potential than adipose derived stem cells(90). A limitation of this technique is the higher morbidity involved in harvesting bone marrow compared to adipose tissue.

As the ear is a structure derived from elastic cartilage, the relative elasticity of formed cartilage constructs is an important consideration for reconstruction. Four studies assessed Young's modulus as a measure of cartilage elasticity with Kang *et al*. and Zhang *et al*. finding co-culture cartilage constructs were most similar to native human cartilage. Kang *et al*. specifically identified a co-culture ratio of 1:1 as most effective at achieving similar elasticity to human cartilage *in vivo*(75,80).

One other potential new consideration for tissue engineering cartilage constructs could be the use of induced pluripotent stem cells (iPSCs) as a source of SCs. iPSCs can be generated from adult cells such as skin or blood cells and have the potential to differentiate into a variety of cell types, including SCs. Using iPSCs, which can be readily derived from abundant adult somatic cells, as a source of SCs could potentially overcome the limitations of using bone marrow or adipose tissue-derived stem cells and the ethical considerations associated with embryonic stem cell use. Additionally, as iPSCs are harvested autologously from the patient, this allows for lower immunogenicity increasing their chance for in-vivo survival. Kim *et al.* found in a 2024 review that iPSC cultures remained limited in its ability to differentiate into complex tissue structures, with larger constructs prone to developing necrotic cores which was not a limitation seen with present chondrocyte mono-culture or co-culture models when creating 3D cartilaginous structures(91). However, it is proposed that when coupled with bioprinting which allows for deposition of iPSCs within 3D scaffolds high fidelity, this limitation could potentially be overcome. None of the studies included in the review utilized iPSCs, with future research into the use of iPSCs potentially allowing for the generation of patient-specific SCs, which could increase the chances of successful tissue engineering.

2.6 Conclusions

Despite fewer chondrocytes required, the chondrogenic potential of co-cultured cells was similar or superior to mono-culture of chondrocytes across the majority of studies which

supports its use in head and neck reconstructive surgery where acquiring donor cartilage is a significant limiting factor. Further studies comparing the efficacy of different co-culture ratios and cell sources will need to be performed to better standardize co-culture techniques prior to translational use in bioinks.

2.7 Acknowledgements

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2.8 Statement of ethics

An ethics statement is not applicable because this study is based exclusively on published literature.

2.9 Conflicts of interest

The authors declare that they have no conflicts of interest, financial or otherwise, related to the research described in the paper.

2.10 Funding

No sources of funding to disclose.

2.11 Figures and tables

Table 1: Comparators used to quantify chondrogenic potential and their measured variables.

Comparator	**Measure**
ECM deposition	ECM presence
Aggrecan/biglycan	ECM component proteoglycans
Glycosaminoglycan (GAG)	ECM component polysaccharide (surrogate marker for proteoglycan)

Elastin/elastic fibres	ECM component protein (elastic cartilage specific)
Lacuna structure	ECM component structure
COL2A1	Chondrogenic gene
SOX9	Chondrogenic gene
ACAN	Chondrogenic gene
Wet weight	Weight/volume
Diameter	Weight/volume
Height	Weight/volume

Table 2: Qualitative summary of included studies. ArC = articular cartilage, AC = auricular cartilage, NC = nasal cartilage, TC = tracheal cartilage, BM = bone marrow (derived stem cells), AT = adipose tissue (derived stem cells), C = chondrocytes, SC = stem cells, Co = co-culture, Mo = mono-culture.

Study (Year)	**Chondrocytes source**	**Stem cell source**	**Maximal duration**	**Ratio**	**Comparators**	***In Vitro***	***In Vivo***
[Acharya *et al*., 2011]	Human ArC Human NC	Human BM Human AT	*In vitro*: 3w	1C:3SC	GAG, COL2A1	**GAG:** CoNC (BM)>CoNC (AT)>MoNC >MoSC **COL2A1:** CoNC>MoSC	
[Anderson-Baron *et al*., 2021]	Human NC	Human BM	*In vitro*: 3w *In vivo*: 3w (post 3w culture)	1C:3SC	GAG, COL2A1, SOX9, ACAN, wet weight	**COL2A1:** MoNC>CoNC>MoSC **SOX9:** MoNC=CoNC>MoSC **ACAN:** MoNC>CoNC>MoSC **Wet weight:** MoNC>CoNC>MoSC	**GAG:** MoNC>CoNC>MoSC
[Apelgren *et al*., 2017]	Human NC	Human BM	*In vivo*: 60d	1C:4SC	GAG		**GAG:** MoNC>CoNC>MoSC
[Cai *et al*., 2015]	Human AC	Human AT	*In vivo*: 8w	3C:7SC	Aggrecan, GAG, lacuna structures, COL2A1, wet weight		**Aggrecan:** CoAC=MoAC>MoSC **GAG:** CoAC=MoAC>MoSC **Lacuna structures:** CoAC=MoAC>MoSC **COL2A1:** CoAC=MoAC>MoSC **Wet weight:** CoAC=MoAC>MoSC
[Cohen *et al*., 2018]	Human AC	Human BM	*In vivo*: 3m	1C:1SC	GAG, lacuna structures, elastin formation, diameter, height		**GAG:** CoAC=MoAC>MoSC **Lacuna structures:** CoAC=MoAC>MoSC **Elastin formation:** CoAC=MoAC>MoSC **Height:** CoAC=MoAC>MoSC **Diameter:** CoAC=MoAC>MoSC
[Goh *et al*., 2016]	Human AC	Human AT	*In vitro*: 3w	Unspecified	Aggrecan, elastin, COL2A1, SOX9	**Aggrecan:** CoAC>MoSC>MoAC **Elastin:** CoAC>MoSC>MoAC **COL2A1:** CoAC>MoSC>MoAC **SOX9:**	

						CoAC>MoSC>MoAC	
[Kang *et al*., 2013]	Porcine ArC	Porcine BM	*In vitro*: 8w	1C:1SC 2C:3SC 3C:7SC 1C:4SC 1C:9SC	GAG, COL2A1, wet weight, diameter, height	**GAG:** MoArC>Co1:1=Co2:3=Co3:7>Co1:4=Co1:9>MoSC **COL2A1:** MoArC=Co1:1=Co2:3=Co3:7>Co1:4=Co1:9>MoSC **Wet weight:** MoArC>Co1:1=Co2:3=Co3:7=Co1:4>Co1:9>MoSC **Diameter:** MoArC=Co1:1=Co2:3=Co3:7=Co1:4>Co1:9>MoSC **Height:** MoArC>Co1:1=Co2:3=Co3:7>Co1:4=Co1:9>MoSC	
[Kang *et al*., 2012]	Porcine AC	Porcine BM	*In vitro*: 8w *In vivo*: 6w (post 8w culture)	1C:1SC 1C:9SC	Biglycan, lacuna structures, elastic fibres, COL2A1, SOX9, ACAN		**Biglycan:** Co1:1=MoAC>CoSC>Co1:9 **Lacuna structures:** Co1:1=MoAC>MoSC=Co1:9 **Elastic fibres:** Co1:1>MoAC>MoSC=Co1:9 **COL2A1:** MoAC>Co1:1>MoSC>Co1:9 **SOX9:** Co1:1=MoAC>MoSC **ACAN:** Co1:1=MoAC>MoSC
[Lv *et al*., 2012]	Porcine AC	Human AT	*In vitro*: 8w	3C:7SC	GAG, lacuna structures, COL2A1	**GAG:** CoAC=MoAC>MoSC **Lacuna structures** CoAC=MoAC>MoSC **COL2A1:** CoAC=MoAC>MoSC	
[Ma *et al*., 2020]	Porcine AC	Porcine AT	*In vitro*: 4w	1C:1SC	COL2A1, SOX9, ACAN	**COL2A1:** CoAC>MoAC>MoSC **SOX9:** CoAC>MoAC>MoSC **ACAN:** CoAC=MoAC>MoSC	
[Moller *et al*., 2017]	Human NC	Human BM	*In vivo*: 60d	1C:4SC	GAG		**GAG:** CoNC>MoNC>MoSC
[Morrison *et al*., 2018]	Porcine AC Porcine TC	Porcine AT	*In vitro*: 4w *In vivo*: 4w (post 4w culture)	1C:1SC 1C:2SC 1C:5SC 1C:10SC	GAG, COL2A1	**GAG:** Co1:1=Co1:2=Co1:5=Co1:10>MoAC	**GAG:** Co1:1=Co1:2=Co1:5=Co1:10=MoAC **COL2A1:** Co1:1=Co1:2=Co1:5=Co1:10>MoAC
[Pleumeekers *et al*., 2015]	Bovine AC Bovine NC	Human BM	*In vitro*: 3w *In vivo*: 8w	1C:4SC	GAG, COL2A1		**GAG:** CoNC=MoNC>CoAC=MoAC>MoSC **COL2A1:** CoNC=MoNC>CoAC=MoAC>MoSC
[Zhang *et al*., 2014]	Human AC	Goat BM	*In vivo*: 12w	1C:3SC	GAG, lacuna structures,		**GAG:** CoAC=MoAC>MoSC

			12 weeks subcutaneous implantation of human ear shaped scaffold with co-cultured cells		COL2A1, wet weight, diameter		**Lacuna structures:** CoAC=MoAC>MoSC **COL2A1:** CoAC=MoAC>MoSC **Wet weight:** CoAC=MoAC>MoSC **Diameter:** CoAC=MoAC>MoSC
[Ziegler *et al*., 2020]	Porcine AC	Human AT	*In vitro*: 10w (note 48h for SOX9 measurement)	1C:5SC 1C:9SC	COL2A1, SOX 9, ECM deposition	**SOX9:** CoAC>MoSC>MoAC **ECM deposition:** Co1:9>Co1:5>MoAC=MoSC **COL2A1:** Co1:9>Co1:5=MoAC=MoSC	

Table 3: Summary of chondrogenic potential comparators between co-culture and mono-culture groups. Co: co-culture, MoC: mono-culture of chondrocytes, MoSC: mono-culture of stem cells.

	Co>MoC	**Co=MoC**	**Co<MoC**	**Co>MoSC**	**Co=MoSC**	**Co<MoSC**	**Referencees**
ECM deposition	1	0	0	1	0	0	Ziegler 2020 [28]
Aggrecan/biglycan	1	2	0	3	0	0	Cai 2015 [17], Goh 2016 [19], Kang 2012 [21]
Glycosaminoglycan	2	6	3	9	0	0	Acharya 2011 [14], Anderson-Baron 2015 [15], Apelgren 2017 [16], Cai 2015 [17], Cohen 2018 [18], Kang 2013 [20], Lv 2012 [22], Moller 2017 [24], Morrison 2018 [25], Pleumeekers 2015 [26], Zhang 2014 [27]
Elastin/elastic fibres	2	1	0	3	0	0	Cohen 2018 [18], Goh 2016 [19], Kang 2012 [21]
Lacuna structures	0	5	0	5	0	0	Cai 2015 [17], Cohen 2018 [18], Kang 2012 [21], Lv 2012 [22], Zhang 2014 [27]
COL2A1	4	5	2	11	0	0	Acharya 2011 [14], Anderson-Baron 2015 [15], Cai 2015 [17], Goh 2016 [19], Kang 2013 [20], Kang 2012 [21], Lv 2012 [22], Ma 2020 [23], Morrison 2018 [25], Pleumeekers 2015 [26], Zhang 2014 [27], Ziegler 2020 [28]
SOX9	3	2	0	5	0	0	Anderson-Baron 2015 [15], Goh 2016 [19], Kang 2012 [21], Ma 2020 [23], Ziegler 2020 [28]
ACAN	0	2	1	3	0	0	Anderson-Baron 2015 [15], Kang 2012 [21], Ma 2020 [23]
Wet weight	0	2	2	4	0	0	Anderson-Baron 2015 [15], Cai 2015 [17], Kang 2013 [20], Zhang 2014 [27]
Diameter	0	3	0	3	0	0	Cohen 2018 [18], Kang 2013 [20], Zhang 2014 [27]
Height	0	1	1	2	0	0	Cohen 2018 [18], Kang 2013 [20]

Table 4: Summary of chondrogenic potential comparators between co-culture and mono-culture groups for studies with auricular chondrocyte sources. Co = co-culture, MoAC = mono-culture of auricular chondrocytes, MoSC = mono-culture of stem cells.

	Co>MoC	**Co=MoC**	**Co<MoC**	**Co>MoSC**	**Co=MoSC**	**Co<MoSC**	**Referencees**
ECM deposition	1	0	0	1	0	0	Ziegler 2020 [28]
Aggrecan/biglycan	1	2	0	3	0	0	Cai 2015 [17], Goh 2016 [19], Kang 2012 [21]
Glycosaminoglycan	0	5	0	5	0	0	Cai 2015 [17], Cohen 2018 [18], Lv 2012 [22], Pleumeekers 2015 [26], Zhang 2014 [27]
Elastin/elastic fibres	2	1	0	3	0	0	Cohen 2018 [18], Goh 2016 [19], Kang 2012 [21]
Lacuna structures	0	5	0	5	0	0	Cai 2015 [17], Cohen 2018 [18], Kang 2012 [21], Lv 2012 [22], Zhang 2014 [27]
COL2A1	3	3	1	6	0	0	Cai 2015 [17], Goh 2016 [19], Lv 2012 [22], Ma 2020 [23], Zhang 2014 [27], Ziegler 2020 [28]
SOX9	3	1	0	4	0	0	Goh 2016 [19], Kang 2012 [21], Ma 2020 [23], Ziegler 2020 [28]
ACAN	0	2	0	2	0	0	Kang 2012 [21], Ma 2020 [23]
Wet weight	0	2	0	2	0	0	Cai 2015 [17], Zhang 2014 [27]
Diameter	0	2	0	2	0	0	Cohen 2018 [18], Zhang 2014 [27]
Height	0	1	0	1	0	0	Cohen 2018 [18]

Figure 1: PRISMA flow diagram demonstrating process of studies being included in this review.

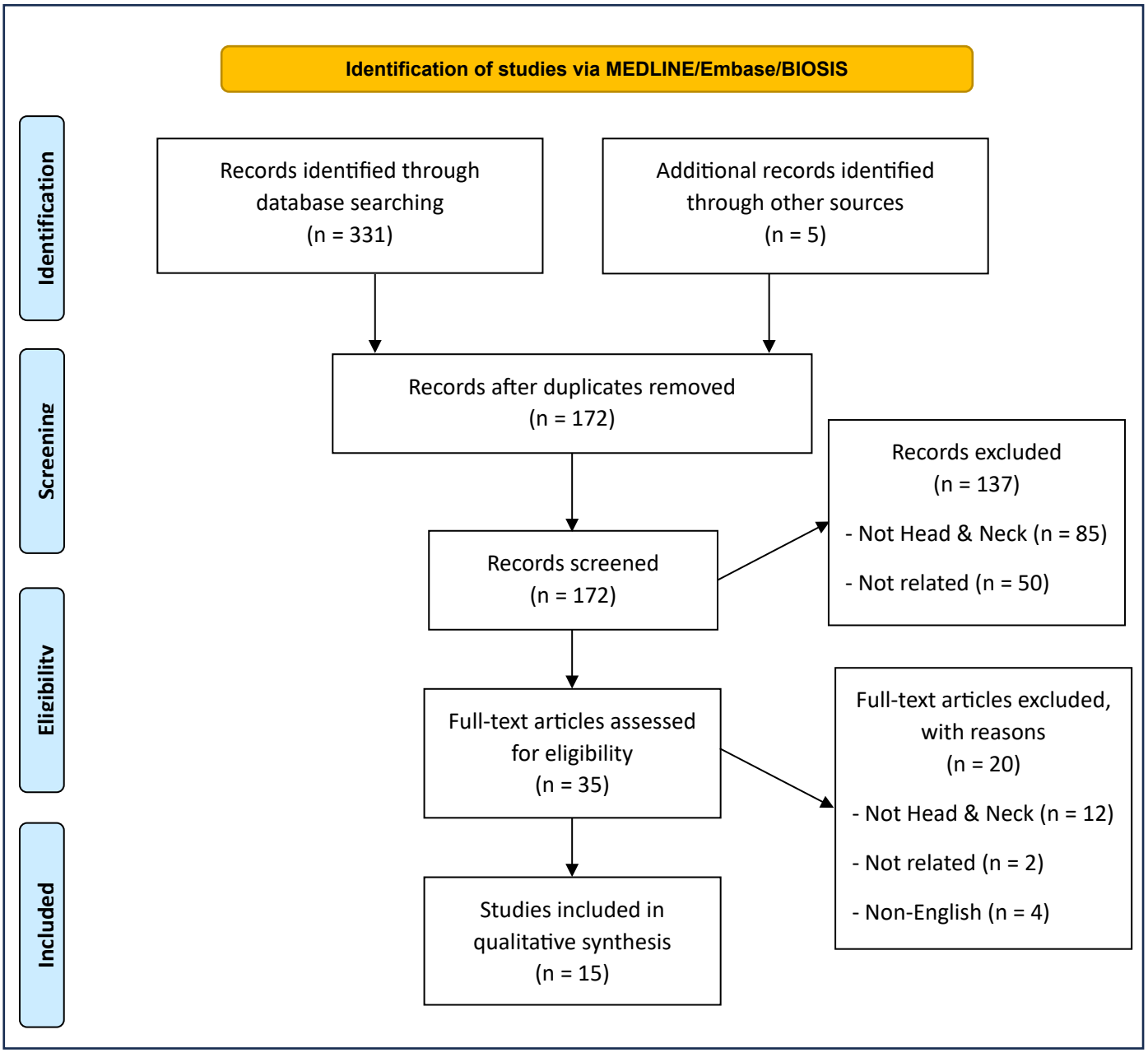
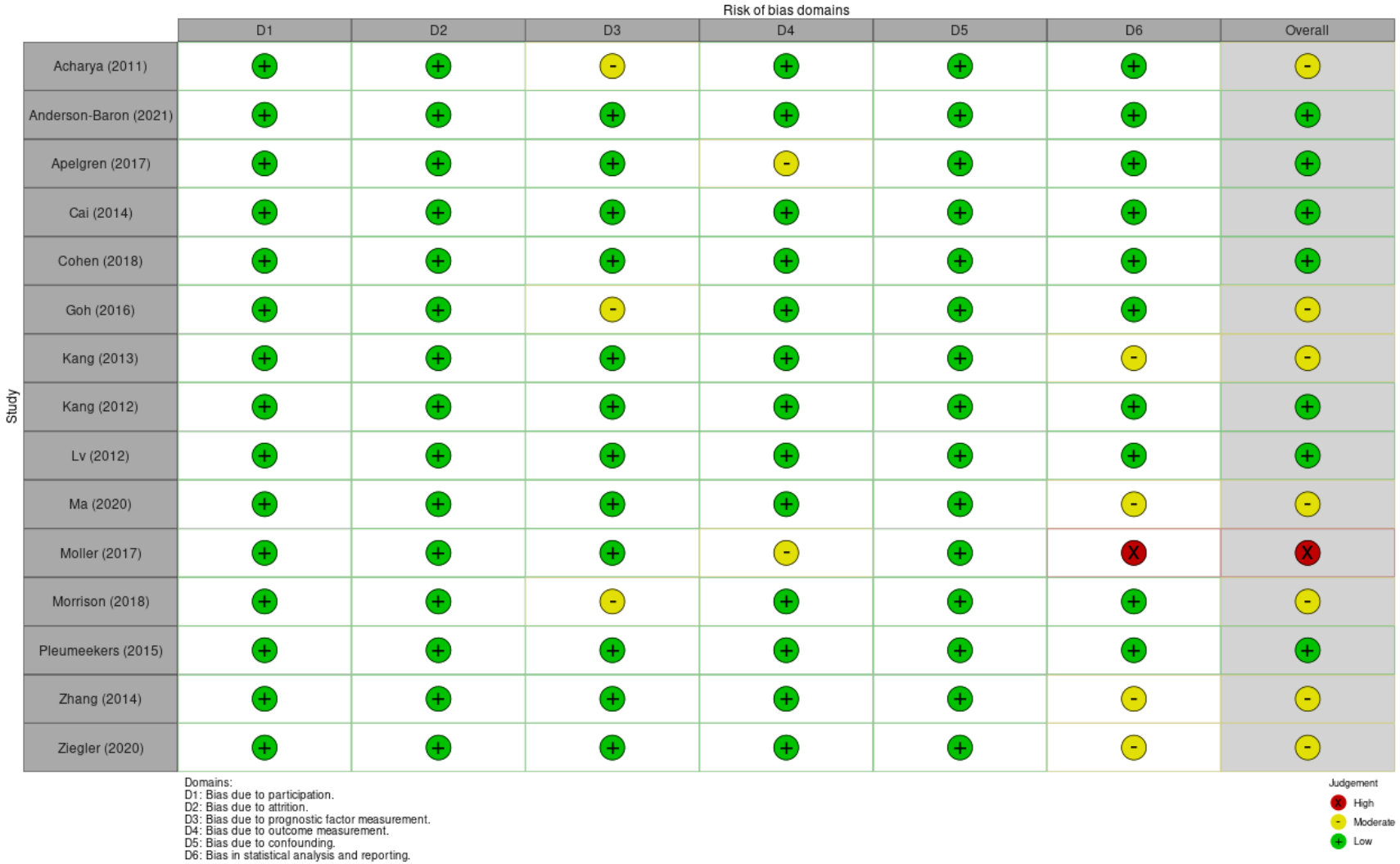


Figure 2: *robvis* representation of QUIPS Risk of Bias assessment.



Chapter 3: Animal study - Chondrocyte harvest viability of auricular and nasal septal cartilage in a sheep model

3.1 Abstract

Introduction: Reconstruction for cartilaginous structures of the head and neck is a challenge, with autologous techniques limited by donor cartilage volume, donor site morbidity and inconsistent results. Alloplastic techniques are limited by implant fracture and extrusion. Bioprinting combines "bioinks" consisting of living cells, supporting structures and biological motifs with a supporting scaffold to create customised implantable constructs. Auricular and nasal septal cartilage are amongst the most viable sources of chondrocytes for bioprinting.

This study reports on the digestion and proliferation results of auricular and nasal septal chondrocytes in a sheep model with the aim of understanding the behavior of different donor sites for chondrocytes and its impact on clinical practice.

Methods: Cartilage was harvested from the ear and nasal septum of six sheep. The cartilage was then digested according to protocol utilising a 0.15% w/v type II collagenase solution in two cycles, with cell counts per gram wet weight calculated and recorded. The digested chondrocytes were seeded at cell densities of 1.5×10^4 for 14 day proliferation, with cell counts calculated and recorded at days 1, 3, 7, 10 and 14.

Results: Auricular and nasal septal chondrocytes yielded an average of 6.09×10^6 and 5.48×10^6 cells per gram of cartilage respectively, with no statistically significant difference between total or viable chondrocyte counts harvested from the two sources post digestion. Nasal septal chondrocytes expanded at a significantly greater rate than auricular chondrocytes, though this was not significant at day 14.

Conclusion: Auricular and nasal septal chondrocytes can be harvested without contamination using an antibiotic solution. There was no significant difference between chondrocytes from the two sources following digestion and 14 day proliferation. This supports both auricular and nasal septal cartilage to be comparable cell sources for use in bioinks. As the two cell sources are comprised of elastic and hyaline cartilage respectively, it is important to consider the intended properties of the formed cartilage when deciding which donor cartilage source to utilise.

Keywords: Bioprinting, reconstructive surgery, chondrocytes, auricular, nasal septal

3.2 Introduction

Cartilage is an important component of many structures of the head and neck, including the pinna, nasal septum, larynx and trachea. As cartilage has a limited capacity for self-regeneration, when congenital or acquired defects of these structures occur, such as due to microtia or ablative oncological surgery, reconstructive surgery can face several challenges.

The current standard of care is autologous reconstruction, whereby native cartilage is harvested from a donor site and moulded to reconstruct the original structure though this is limited by the availability of sufficient donor cartilage, donor site morbidity and requires a high degree of surgical precision resulting in inconsistent aesthetic results(57–59). Alloplastic implants can more consistently match complex 3D structures in appearance but are limited by high rates of fracture and extrusion(60).

One emerging reconstructive technique that addresses these limitations is bioprinting wherein living cells can be delivered alongside a 3D printed structural support customised to individual patients. Contemporary bioprinting techniques typically involve the creation of a biodegradable scaffold for "bioinks" consisting of living cells, supporting structures such as hydrogel matrices and additional biological motifs such as growth factors(44). In recent years, this had been popularised through research into bioprinted organs such as the pancreas utilising pancreatic islets or the liver utilising hepatocytes as living cells in bioinks(92,93).

As the only cell line in cartilage, chondrocytes have seen use in preclinical studies as the living cell type in bioinks for several 3D printed head and neck structures including the pinna, nasal septum, and trachea(94–96). For clinical relevance, cartilage should ideally be harvested from a local site, to reduce the morbidity involved from creating a secondary donor site(57). Additionally, the volume of autologous cartilage likely to be harvested will be limited, with additional proliferation via cell culture required. In the head and neck, the pinna and nasal septum are amongst the most readily accessible sources of cartilage with cartilage harvest from these sources already being a standard of care in several procedures including myringoplasty, septal reconstruction, rhinoplasty, ossicular chain reconstruction and repair of skull base defects(21,97–99). Tissue engineered constructs containing auricular or nasal septal chondrocytes seeded in scaffolding are already being used in pre-clinical studies to create pinna

shaped structures for implantation(88,94). However, the inability of chondrocytes to produce perichondrium, which plays a vital role in maintaining and providing a blood supply to cartilage, remains a limitation of tissue engineered cartilage(100).

This study reports on the cell digestion and proliferation results following chondrocyte harvest from auricular and nasal septal sources in a sheep model. The objective is to understand the behavior of different donor sites for chondrocytes and its impact on clinical practice.

3.3 Methods

a. Study design and ethical oversight

The animals used in this study were approved for cartilage harvest with ethics approval from the University of Sydney Animal Ethics Committee (2021/2004) in accordance with the New South Wales, Australia Animal Research Act (1985).

b. Animal model

Auricular and septal cartilage were harvested from six healthy skeletally mature sheep. Selected sheep had no pre-existing cartilaginous deformity of the ear or nose. The sheep harvest was performed at the Charles Perkins Centre after induction of general anaesthesia by an animal anesthetist.

c. Operative procedure

Following general anaesthesia, the sheep ear and nasal septum were injected with a local anaesthetic solution containing 1% lignocaine with 1:100,000 adrenaline targeting the subperichondrial plane to aid with analgesia, hemostasis and hydrodissection. Using a sterile 15 blade, an incision was made to the sheep ear near the root of the concha down to cartilage. A Freer periosteal elevator was used to dissect the skin and perichondrium layers off the auricular cartilage, following which a 1 x 2 cm segment was removed. The root of the concha was chosen as the skin closer to the ear tip was found to be densely adherent to the underlying perichondrium and cartilage limiting ease of dissection. A fresh, sterile 15 blade was used to perform a left hemitransfixion incision to the nasal septum exposing the anterior quadrilateral cartilage of the septum. A Freer periosteal elevator was used to separate the perichondrium

layers off the quadrilateral cartilage. The anterior 1cm of the nasal septum was preserved, in-line with human septoplasty practice, where this segment known as the "L-strut" is preserved to maintain structural integrity of the nose. A 1 x 2 segment of the quadrilateral cartilage was isolated and removed. Following cartilage harvest, both ear and nasal wounds were closed with dissolvable vicryl suture. The segments of auricular and nasal septal cartilage were diced into smaller samples approximately $1mm^3$ to facilitate subsequent digestion. Immediately after dicing, the cartilage pieces were placed into labelled 20mL sterile specimen containers filled with 10mLs of a 10% v/v penicillin-streptomycin solution (10,000 U/mL, Gibco) (P/S).

d. Cell digestion

Following operative harvest, the cartilage pieces were transferred to the RPA Institute of Academic Surgery for further processing. Each cartilage specimen was placed into a sterile pre-weighed Petri dish with the net weight recorded. Phosphate buffered saline (PBS) solution supplemented with P/S was used to rinse the cartilage pieces twice. Following PBS rinse, the cartilage pieces were placed in 50mL Falcon tubes containing a cartilage digestion media (Ham's F-12K nutrient mixture (ThermoFisher), 10% foetal bovine serum (FBS, Interpath) + 0.15% (w/v) collagenase type II) targeting a 10% digestion ratio. The solution was thoroughly mixed, following which they were kept for digestion overnight on an inverter at 37°C and 5% CO_2. The digested specimens were then filtered through a 100µm strainer into 50mL Falcon tubes and labelled as the 1st digestion. The unfiltered residue was then returned to fresh 50mL Falcon tubes containing cartilage digestion media for a repeat overnight digestion and a subsequent filtration was performed and labelled as the 2nd digestion. For both the 1st and 2nd digestion, following overnight digestion the filtrate was centrifuged at 700*g* for 5 minutes followed by aspiration of the supernatant and re-suspension in PBS solution, with the process repeated three times in total. Subsequently, the cells were suspended in a chondrocyte expansion medium containing Ham's F-12K nutrient mixture, 10% foetal bovine serum and 1% v/v P/S then counted using a Countess 3 Automated Cell Counter (Invitrogen)with trypan blue staining(101). The remaining cells were then seeded into a T25 flask, grown and subcultured to passage 2 (P2) ahead of proliferation. Passaging was achieved using 1mL of 0.25mM trypsin (Gibco) to detach cells followed by centrifugation at 1000rpm for 3 minutes to separate from supernatant, then resuspended and replated onto T75 flasks for subcultures.

Gene analysis performed by real-time polymerase chain reaction (qPCR) was used to verify the cells as chondrocytes.

e. Cell proliferation

After digestion, the cells were transported to the Intelligent Polymer Research Institute (IPRI) at the Australian Institute for Innovative Materials (AIIM) facility at the University of Wollongong (UoW), where chondrocytes and remaining cartilage were placed in a humidified incubator at 37ºC, with 5% CO_2 for cell proliferation. Live chondrocytes were plated into individual 6-well plates at cell densities of 1.5 x 10^4 cells/cm^2 (equivalent to 1.425 x 10^5 cells/well). 4 plates in total were created respectively for each chondrocyte line, either auricular or nasal septal, and for each sheep respectively resulting in a total of 48 plates. 1mL of 0.25 mM trypsin (Gibco) was used to detach cells from the plate, with cell counts performed using a Countess 3 Automated Cell Counter at day 1, 3, 7, 10 and 14.

f. Statistical analysis

Statistical analyses were performed using Graph Pad Prism 9.0.0 (GraphPad, La Jolla, CA, USA). Grubb's Test was used to identify and remove outliers. When data sets adhered to normal distribution, One-Way or Two-Way ANOVA with Tukey's multiple comparisons test for multiple comparisons were used.

3.4 Results

Operative cartilage harvest and cell digestions were successfully completed on all six sheep. There were no difficulties isolating the 1 x 2cm segment of cartilage from either the ear or nose in any of the sheep with cell digestion performed on all twelve specimens.

a. Cell digestion

The weight and cell count of harvested cartilage are summarised in table 1, with auricular cartilage weighing an average of 0.32g and nasal septal cartilage weighing an average of 0.24g. A ratio for the average amount of cells per gram of harvested cartilage was calculated by combining the first and second cell digestion counts and dividing by the wet weight of the original cartilage specimen. Figure 1 shows the total and live auricular (left) and septal (right) chondrocytes harvested following two digestions per gram of cartilage. Whilst more auricular

chondrocytes were harvested, there was no significant difference in total or viable (live) chondrocytes harvested between auricular and septal cartilages. There were no instances of contamination amongst the specimens.

b. Cell proliferation

For each sheep, there were four plates created for either auricular or nasal septal chondrocytes respectively, with the mean cell count of these four plates used in comparative analysis. All plates irrespective of sheep or cell source were seeded at an initial density of 1.5×10^4 cells/cm^2 at day 0.

Homogenous cell number seeding onto 6-well plates was confirmed by examining day 1 cell numbers, which showed that wells had similar initial cell densities and survival rates (viability). When auricular and septal chondrocytes were cultured on plastic surfaces, septal chondrocytes expanded at a significantly greater rate compared to auricular (Figure 2). However, by day 14 there was no significant difference in cell density between these two groups.

Interestingly, when compared to day 14 human septal chondrocytes (Figure 3, unpublished data), there is also no significant difference observed across these groups.

3.5 Discussion

With the aim of comparing auricular and nasal septal chondrocyte sources to function as the living cell line for bioinks in 3D bioprinted constructs for the head and neck, this study assessed the cell count yielded both from digestion and subsequent proliferation of cells from the two groups.

The sheep is a validated animal model for this study, owing to similarities in the anatomy of the ear and nose in comparison to humans, and the large animal size allowing for harvest of cartilage in greater quantities than would be possible for smaller animal models(102,103).

Auricular and nasal septal cartilage were easily accessible for harvest from their respective donor sites. The methods of cartilage harvest were like those conventionally performed in human ear and nasal septal surgery and being an animal model with similar anatomy to humans,

this lends towards the translatability of this approach for future studies performing human cartilage harvest. This study is the first to detail a protocol for cartilage harvest from the ear and nose in a sheep model. The native flora of the nose and ear are similar, with gram positive bacteria including staphylococcus epidermis, diphtheroids, and staphylococcus aureus being the most common organisms(104,105). For the nose, there is a low but significant rate of gram negative colonisation as well, most commonly haemophilus influenzae(104). Penicillin-streptomycin was an ideal choice for our study, as a well validated for antibiotic solution for chondrocyte preparation with both gram-positive and gram-negative coverage and use in both human and animal settings(106,107).

For the digestion phase, we utilised type II collagenase as the enzymatic digestion agent as previously described in the literature and two digestion phases to maximize the cell yield(108). Both collagenase and trypsin are well described in the literature, with studies findings differences in digestion time but no significant difference in cell yield(109). Our findings of 6.09×10^6 and 5.48×10^6 cells per gram for the auricular and nasal septal cartilage respectively was higher than similar studies in the literature which found between $1.0 – 1.5 \times 10^6$ cells per gram of cartilage in a sheep study utilising nasal septal cartilage(108). No existing studies examining sheep auricular cartilage reporting on cell yield per gram wet weight was available for direct comparison. Whilst the average cell yield per gram of auricular cartilage was higher than nasal septal cartilage, the difference was not statistically significant.

Following proliferation, nasal septal chondrocytes consistently demonstrated higher cell counts than auricular chondrocytes following initial seeding at the same density (1.5×10^4 cells/cm^2), however with this difference plateauing by day 14. As this study aims to assess the suitability of the two cell sources to maximise cell numbers for bioprinting applications, we sought to assess speed of cell growth through measuring total cell numbers and viability at specific timepoints rather than population doubling time. Therefore, Day 14 was chosen as the endpoint of our study as cells would be approaching their confluence at this time. Our findings are similar to those of Hellingman *et al*. and Idrus *et al*., who both found significantly higher rates of proliferation with nasal septal chondrocytes compared to auricular chondrocytes in initial cell passages, but no difference with subsequent passages (110,111). These findings support the viability of chondrocytes from both auricular and nasal septal sources as a potential living

cell line for 3D printed cartilage constructs. It is interesting to note that chondrocytes isolated from articular cartilage, which like the nasal septum is hyaline in nature, also proliferated at a rate closer to nasal septal chondrocytes than to auricular chondrocytes(110,112). While it has been theorised that differences in gene expression between chondrocytes of different origins may contributes towards the different nature of formed cartilage, in a direct comparison between articular, nasal septal and auricular chondrocytes, Idrus *et al.* found no differences in gene expression by PCR between the three groups through three passages of digestion(110).

Differences in gene expression profiles between the two cell sources may also affect the quantity of effective cartilage produced despite similar proliferation profiles. Hellingman *et al*. and He *et al*. both found that despite auricular chondrocytes tending towards slower proliferation, cartilage pellets produced were both larger and more closely reapproximated normal cartilage than those produced by nasal septal chondrocytes(111,113). It was proposed this may be due to a higher expression of growth factors with an anabolic effect on cartilage matrix productions in auricular chondrocytes and a higher expression of enzymes involved in cartilage matrix degradation in nasal septal chondrocytes(111).

a. Impact on clinical translation in the head and neck

Classically, articular cartilage is the most common source of chondrocytes for tissue engineering purposes, being readily available from a number of sites such as the proximal tibia or femur(114). For reconstructive surgery of the nose such as rhinoplasty, autologous cartilage is preferentially harvested locally from the nasal septum or when insufficient, costal cartilage from the rib which is similarly hyaline in nature with comparable rigidity and compression resistance(98,115). Comparatively, for reconstruction of outer and middle ear structures such as in myringoplasty and partial pinna reconstruction, cartilage is preferentially harvested locally from auricular sources containing elastic cartilage such as the tragus, antihelix or conchal bowl(97,116,117). Auricular cartilage has previously been proposed as a source of cartilage especially in revision rhinoplasty where there is a shortage of autologous septal cartilage and to bypass the morbidity of costal cartilage harvest(118,119). However, owing to its elastic rather than hyaline nature, auricular cartilage grafts are more prone to fibrosis and loss of stability over longer term follow-up(115,120). Similarly, when utilising hyaline cartilage from the nasal septum or rib, its rigid nature and lower malleability differs

significantly from native auricular cartilage affecting its suitability for pinna reconstruction(116). In a direct comparison between costal and auricular cartilage, Griffin *et al*. found a significantly lower Young's elastic modulus for auricular cartilage constructs compared to costal cartilage and alloplastic materials such as silicone, polytetrafluoroethylene and porous polyethylene(121). Whilst the difference in mechanical properties suggests hyaline cartilage is a poor mechanical match for pinna reconstruction, it has also been proposed the increased rigidity may actually help implants withstand shape deformation, with one of the primary causes of long term failure of autologous reconstruction being implant warping due to compression from the overlying skin (29,34). For reconstruction elsewhere in the ear such as myringoplasty wherein the elastic nature of auricular cartilage is less important, nasal septal cartilage grafts have been safely utilised with good outcomes and may avoid the unnecessary creation of a donor site when conducted alongside an indicated septoplasty(122). As both are readily accessible donor sites with low morbidity, both the ear and nasal septum have been described as donor cartilage sources for reconstruction of other structures in the head and neck such as the orbital floor and in laryngotracheal reconstruction(123,124).

It is important to note that despite similar digestion and proliferation profiles, auricular and nasal septal cartilage are morphologically different. Auricular cartilage is elastic in nature and contains elastic fibres in addition to type II collagen, whilst nasal septal cartilage consists primarily of type II collagen with only low amounts of elastin(125). This difference is relevant when considering the most appropriate source of cartilage for reconstruction of a given structure, in order to re-approximate its native properties. It has also been proposed that auricular chondrocytes exhibit a better response than nasal septal chondrocytes to introduced growth factors, allowing for improved proliferative rates and *in vitro* cartilage implant growth(126). Further studies on the different structural and biochemical differences between constructs formed by cells of different lineages will help establish clinical scenarios where auricular and nasal septal chondrocytes can be used interchangeably.

In approaching clinical translation, Velasquillo *et al*. found that chondrocytes harvested from remaining human microtia tissue combined with a 3D polycaprylactone auricle framework was able to create a pinna structure *in vivo* with this technique holding potential for use in autologous reconstruction. It was noted that formed constructs maintained their shape well and

demonstrated similar histochemical characteristics as native auricular cartilage(94). Similarly, Shokri *et al*. found using an *in vivo* rabbit model that auricular chondrocytes combined with an elastin-gelatin-hyaluronic scaffold was able to effectively regenerate a nasal septal cartilage defect, additionally noting autologous chondrocytes performed better than homologous ones(95). Whilst this study did not form cartilaginous constructs to compare the mechanical properties of the two cell sources, differences in strength and flexibility may be less relevant when used in cell-laden bioinks for 3D bioprinting where these properties could be provided by scaffolding material. Chung *et al.* found the use of polycaprolactone (PCL) as a structural support for a human stem cell-laden hydrogel was able to create 3D printed structures with mechanical properties closely resembling human auricular cartilage(127).

A concern for surgeons for using nasal septal cartilage as a donor graft is bacterial or viral contamination given that the septal mucosa in an unsterile environment(128). However, our study demonstrates that handling this cartilage in an antibiotic solution prevents contamination of the chondrocytes prior to digestion. Therefore, while septal cartilage grafts may not currently be routinely used in the ear or other areas of the head and neck, once cultured, nasal septal chondrocytes could be extremely versatile for use in a wide range of defects.

b. Impact on clinical practice

For good long term outcomes of chondrogenesis, there are 3 main factors that need to be addressed: cell count, growth factors and host environment. A higher primary chondrocyte count will facilitate better chondrogenesis. However, a limitation remains when donor chondrocytes cannot be harvested in sufficient quantities, regardless of auricular or nasal septal sources, for example in cases of high-grade auricular dysplasia in grade II – III microtia or in revision rhinoplasty where removal of further septal cartilage risks destabilisation of the nose. One proposed solution to insufficient chondrocyte volumes is co-culture wherein stem cells, which are pluripotent in nature, can exhibit induced chondrogenic activity if cultured alongside chondrocytes(47,49). Posniak *et al*. utilised a human nasal septal chondrocyte and bone marrow-derived stem cell co-culture combined with a 3D polycaprylactone scaffold to produce constructs with comparable cell proliferation and chondrogenic expression as compared to culture of chondrocytes alone at the same cell density(88).

However, limited research has been conducted into regenerating cartilage using the most supportive host environment for the head and neck region, which the results presented in this study help support. A novel potential solution is to utilise the body itself as an incubator to grow cartilage and perichondrium for future use. The perichondrium is a dense, vascularised connective tissue that surrounds both elastic and hyaline cartilage and plays a vital role in the maintenance of cartilage and contributes towards its innate albeit limited capacity to regenerate after injury. Comprised of an inner and outer layer, the inner layer (cambium) contains chondroblasts that differentiate into chondrocytes in the formation of new cartilage, whilst the outer fibrous layer contains fibroblasts which produce collagenous fibres(100,103). The role of perichondrium is especially vital in septal reconstruction, where it has been shown to contribute to 25% of the bending strength of cartilage, and in tympanoplasty where composite grafts containing both cartilage and perichondrium aid in the repair of larger perforations(102,129). Whilst chondrocyte cultures can produce cartilaginous structures, the formed cartilage is avascular in nature and lacks a perichondrium layer leading to reduced capacity for self-repair. This is clinically relevant in reconstructing structures such as a pinna, where the lack of a vascular perichondrium layer increases the risk of failure due to necrosis of tissue-engineered cartilaginous implants(130). Given the interchangeable nature of auricular and nasal septal cartilage for several reconstructive purposes and similar proliferative profiles seen in this study, it is proposed that chondrocytes supported with growth factors can be placed for incubation inside the body to grow cartilage for future operations, such as revision rhinoplasty or as part of a staged pinna reconstruction for microtia. As an easily accessible cavity with low morbidity, the nasal septum may be an ideal environment for this purpose, and anatomically being sandwiched between two layers of perichondrium may even allow for neocartilage to develop its own perichondrium layer. Further studies into the efficacy of techniques such as co-culture for chondrocyte population expansion and cartilage formation using the body as an incubator will better inform our ability to engineer cartilage for use in head and neck reconstruction.

Primary study limitations were the small sample size and being an animal study, the lack of generalisability to humans. The study only evaluated the cell counts post proliferation on culture plates, and not the size of formed pellets or histologic and biochemical differences between the two cell sources. This limits our ability to determine how the cells culture on different scaffold materials or compare the mechanical properties of the formed pellets. Whilst

this study establishes an effective protocol for cell harvest, digestion and proliferation protocol, future studies utilising this protocol in conjunction with different hydrogel bioinks and scaffolding to compare the mechanical properties of formed constructs are required to translate our findings for use in 3D bioprinting. Another limitation was that digested cartilage could not be reweighed prior to the second digestion, and therefore whether the second digestion was more successful or not in releasing a greater concentration of cells per gram of cartilage is unknown. This was due to the presence of excess digestion media that could not be removed without inadvertently also removing or killing cells. Moreover, as only the wet weight was recorded, weight variation between samples could be attributed to water weight rather than cell density. Future experiments should consider acquiring cartilage for dry weight analysis, subsequent quantitative biochemical testing (such as a PicoGreen assay) to determine DNA content of samples and the use of flow cytometry to compare cell population activity within cultures.

3.6 Conclusions

Auricular and nasal septal chondrocytes can be harvested from sheep ear and nasal septal cartilage without contamination using an antibiotic solution. There was no significant difference in the total or viable chondrocyte counts harvested from the two sources post digestion. Nasal septal chondrocytes expanded at a significantly greater rate than auricular chondrocytes, though this was not significant at day 14. This supports both auricular and nasal septal cartilage to be comparable cell sources for use in bioinks. As the two cell sources are comprised of elastic and hyaline cartilage respectively, it is important to consider the intended properties of the formed cartilage when deciding which donor cartilage source to utilise.

3.7 Acknowledgements

The authors would like to acknowledge the support of the Laboratory Animal Services team in conducting the animal study at the Charles Perkins Centre.

3.8 Statement of ethics

This study was conducted in line with the University of Sydney Animal Ethics Committee (2021/2004) and New South Wales, Australia Animal Research Act (1985). Human septal cartilage was harvested with consent provided and ethics approved (HREC 2018-023).

3.9 Conflicts of interest

The authors declare that they have no conflicts of interest, financial or otherwise, related to the research described in the paper.

3.10 Funding

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3.11 Figures and tables

Table 1: Cartilage harvested wet weight and cell counts following digestion

	Cartilage Harvested (g)	**Cell count (1st Digestion)**	**Cell count (2nd Digestion)**	**Average cells/g (wet weight)**
Sheep 1 (Ear)	0.38	922500	1657500	6789473.68
Sheep 1 (Nose)	0.11	940000	58500	9077272.73
Sheep 2 (Ear)	0.26	1525000	453500	7609615.39
Sheep 2 (Nose)	0.31	58500	117250	566935.48
Sheep 3 (Ear)	0.57	2565000	1145000	6508771.93
Sheep 3 (Nose)	0.31	512750	88000	1937903.23
Sheep 4 (Ear)	0.34	470250	922500	4096323.53
Sheep 4 (Nose)	0.3	425250	205250	2101666.67
Sheep 5 (Ear)	0.15	43900	395750	2931000.00
Sheep 5 (Nose)	0.15	396500	146500	3620000.00
Sheep 6 (Ear)	0.21	927000	663000	7571428.57
Sheep 6 (Nose)	0.26	4370000	569000	18996153.85

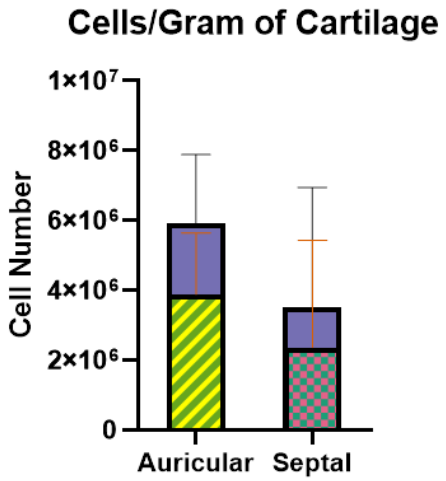

Figure 1: Total (purple) and live cells of sheep auricular (stripes, n = 6) and septal (checkers, n = 6) chondrocytes harvested from cartilage biopsies. Bar charts represent average of total and live cells harvested per gram of cartilage. Error bars represents standard deviation between replicates and significance was calculated with Two-Way ANOVA and corrected using Tukey's multiple comparison test.

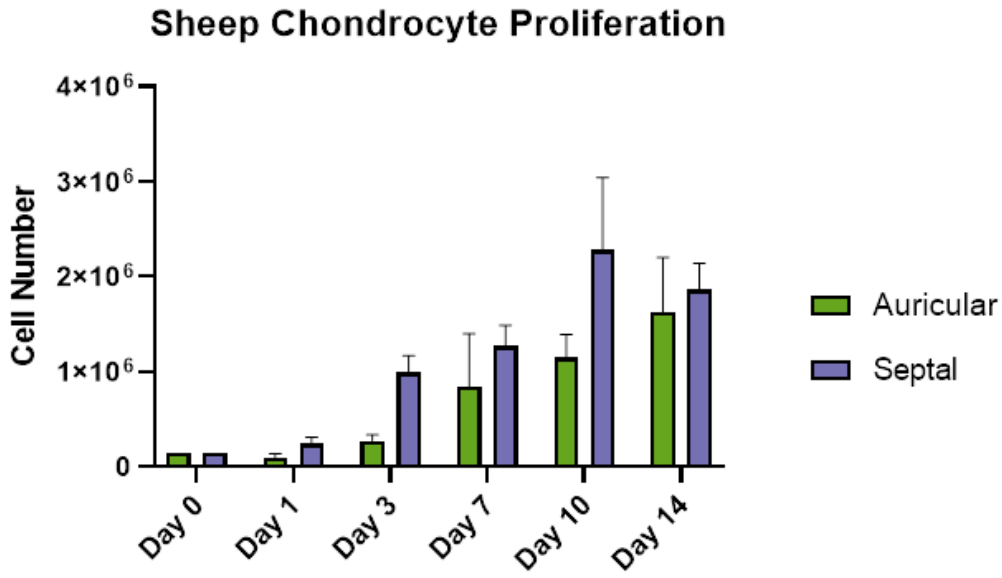

Figure 2: Proliferation of sheep auricular (green, n = 6) and septal (purple, n = 5) chondrocytes over a 14-day period. Grubbs Test was used to identify and remove any outliers. Bar charts demonstrates statistical significance of cell growth in vitro. Error bars represents standard deviation between replicates and significance was calculated with Two-way ANOVA and corrected using Tukey's multiple comparison test, where: * = <0.05, ** = <0.01, *** = <0.001 and **** = <0.0001. See Appendix #1 for statistical significance.

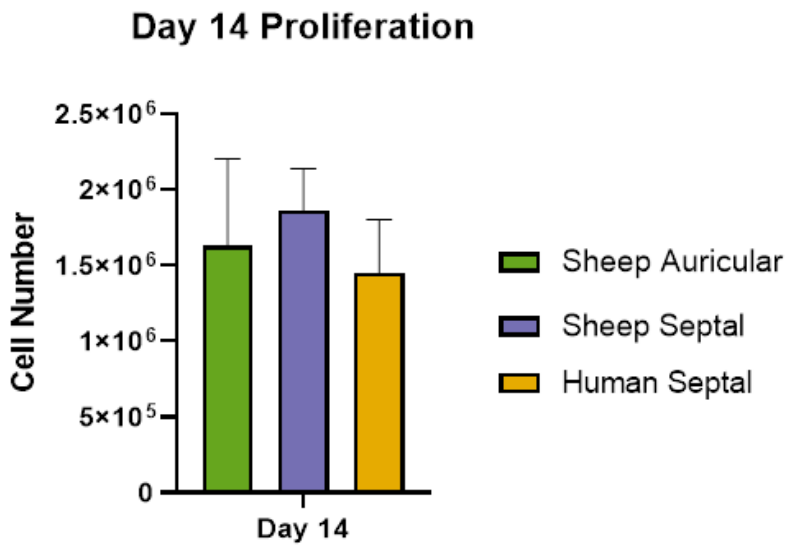

Figure 3: Comparison of day 14 expanded chondrocytes of sheep auricular (green), sheep septal (purple) and human septal (yellow). Grubbs Test was used to identify and remove any outliers. Bar charts demonstrates statistical significance of cell growth in vitro. Error bars represents standard deviation between replicates and significance was calculated with One-Way ANOVA and corrected using Tukey's multiple comparison test.

3.12 Appendix

Appendix 1: Sheep chondrocyte proliferation statistical significance

Tukey's multiple comparisons tests	Mean diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted p value
Auricular chondrocytes					
0 vs. 1	48915	8018 to 89813	Yes	*	0.0178
0 vs. 3	-128591	-195754 to -61427	Yes	***	0.0006
0 vs. 7	-698167	-1247623 to -148710	Yes	*	0.0114
0 vs. 10	-1001091	-1253216 to -748966	Yes	****	<0.0001
0 vs. 14	-1487138	-2051775 to -922500	Yes	****	<0.0001
1 vs. 3	-177506	-240556 to -114457	Yes	****	<0.0001
1 vs. 7	-747082	-1330404 to -163760	Yes	*	0.0115
1 vs. 10	-1050006	-1301950 to -798062	Yes	****	<0.0001
1 vs. 14	-1536053	-2075635 to -996471	Yes	****	<0.0001
3 vs. 7	-569576	-1217934 to 78782	No	Not significant	0.0947
3 vs. 10	-872500	-1112429 to -632571	Yes	****	<0.0001
3 vs. 14	-1358547	-1822643 to -894450	Yes	****	<0.0001
7 vs. 10	-302924	-947381 to 341533	No	Not significant	0.5978
7 vs. 14	-788971	-1716294 to 138352	No	Not significant	0.1118
10 vs. 14	-486047	-963904 to -8189	Yes	*	0.0457
Nasal septal chondrocytes					
0 vs. 1	-95633	-180560 to -10706	Yes	*	0.0274
0 vs. 3	-844389	-1060518 to -628259	Yes	****	<0.0001
0 vs. 7	-1126889	-1386923 to -866855	Yes	****	<0.0001
0 vs. 10	-2135950	-2991573 to -1280327	Yes	****	<0.0001

0 vs. 14	-1719450	-2030430 to -1408470	Yes	****	<0.0001
1 vs. 3	-748756	-989252 to -508259	Yes	****	<0.0001
1 vs. 7	-1031256	-1281345 to -781166	Yes	****	<0.0001
1 vs. 10	-2040317	-2999892 to -1080741	Yes	***	0.0005
1 vs. 14	-1623817	-1951603 to -1296030	Yes	****	<0.0001
3 vs. 7	-282500	-676550 to 111550	No	Not significant	0.1857
3 vs. 10	-1291561	-2311376 to -271746	Yes	*	0.0143
3 vs. 14	-875061	-1295064 to -455058	Yes	***	0.0006
7 vs. 10	-1009061	-1824881 to -193242	Yes	*	0.0164
7 vs. 14	-592561	-756113 to -429009	Yes	****	<0.0001
10 vs. 14	416500	-547265 to 1380265	No	Not significant	0.6536

Chapter 4: Conclusions

4.1 Summary of findings

The history of cartilaginous reconstruction of the head and neck is extensive and dates back as far as 600 BCE. In this time pioneers have proposed techniques ranging initially from local flap-based reconstruction to autologous and alloplastic reconstructive techniques. Whilst autologous techniques were initially multistage procedures, innovations by modern surgeons have allowed autologous reconstruction of structures such as the ear to occur in as little as a single stage procedure but the aesthetics and viability of the result remains largely surgeon skill dependent.

Bioprinting represents one of the newest proposed solutions to rebuilding cartilaginous defects in the head and neck, made possible through innovations in 3D data acquisition using computed tomography and magnetic resonance imaging, as well as the development of 3D bioprinters. If utilised effectively, bioprinting could allow the consistent creation of 3D cartilaginous structures for implantation, customized to each specific patient.

Through contemporary engineering efforts, bioprinters now exist capable of extrusion printing precise scaffolding with the inclusion of bioinks. This thesis highlights some of the considerations in determining an ideal living cell line, one of the three constituent components of bioinks.

One of the primary limitations of autologous reconstructive techniques is the ability to harvest sufficient donor cartilage. For the head and neck, this is ideally harvested from a local source, for example nasal septal cartilage for use in rhinoplasty or the hypoplastic pinna for microtia reconstruction. The challenge is abundantly clear when considering the microtia case, how is it possible to acquire sufficient cartilage from a hypoplastic pinna to reconstruct and create a fully-formed pinna.

Our literature review found that through a process known as co-culture, auricular chondrocytes were able to induce chondrogenic activity in stem cells to create structures with equivalent or greater chondrogenic expression and weight/size as auricular chondrocytes alone at the same cell density. This finding was also similar when utilizing chondrocytes of alternate head and neck lineage including nasal septal and tracheal cartilage. As stem cells can be abundantly sourced with low morbidity from bone marrow or adipose tissue sources, the efficacy of co-

culture as reported in the literature lends to its' ability as a technique for expansion of chondrocyte populations, potentially allowing chondrocytes harvested from a given quantity of cartilage to produce a larger cartilaginous structure when co-cultured with stem cells(47,49). For future clinical scenarios where reconstruction of a cartilaginous defect requires a large amount of chondrocytes to be harvested, the addition of stem cells in co-culture could decrease the amount of native chondrocytes required and therefore reduce donor site morbidity, or potentially enable the use autologous cartilage alone altogether in cases where donor cartilage would otherwise by insufficient.

Our animal study sought to qualify a digestion protocol for chondrocyte harvest from the ear and nose ahead of subsequent studies utilizing harvested chondrocytes as part of bioinks. Our protocol which utilised 0.15% w/v type II collagenase was effective and easily reproducible with consistent results, successfully isolating higher chondrocytes counts per gram of cartilage even when compared to existing literature supporting its future use in our studies. The use of a penicillin-streptomycin solution was also supported with no loss of chondrocytes due to contamination in the digestion phase. Additionally, auricular and nasal septal cartilage were demonstrated to have similar rates of proliferation with no statistically significant difference between the two groups, supporting the use of both cell sources depending on the need for elastic or hyaline cartilage respectively.

4.2 Future directions

The findings of this thesis also highlight several unexplored areas in the existing literature that should be explored in the future to better guide living cell line choices for bioinks.

Whilst co-culture has been demonstrated to be effective for expansion of effective chondrocyte populations, the ratios of chondrocytes to stem cells remains irregular (ranging from 1:1 to 1:9 in the review). Additionally, only one study to date by Posniak *et al*. has explored the use of co-cultured cells specifically as a bioink in 3D bioprinting. Further studies utilising different co-culture ratios and comparing the bioactivity and weight/size of formed constructs will better guide the use of this technique in the future and may allow for volumetric calculations of cell count requirements in creating a construct of a given size. Additionally, it is proposed that future studies placing chondrocytes supported by growth factors inside the body may allow for

the growth of cartilage for use in subsequent operations and in the right environment, may potentially support the development of perichondrium.

Many of the studies included in the review utilised cartilage from hyaline (nasal septal, articular) and elastic (auricular, tracheal) sources interchangeably. Whilst no direct comparison between the mechanical properties of the two groups could be made in our animal study, the literature supports the cartilage formed tends to maintain the hyaline or elastic properties of its donor source, with the biggest difference being hyaline cartilage having increased rigidity compared to elastic cartilage. For future clinical use, the decision on cartilage source will depend on what properties are desirable for the graft, with our study demonstrating that both nasal septal and auricular chondrocytes could be readily harvested with similar digestion and proliferation profiles using our protocol. Additionally, the use of supporting scaffolding in bio-printing could help overcome mechanical limitations of different cell sources.

The use of the body itself as an incubator with the aim of helping neocartilage develop a perichondrium covering is a novel idea proposed in our animal study. If a perichondrium covering can be successfully engineered, this could help further allow for creation of cartilage grafts with high bending strength and ability for self-repair.

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Appendix

Systematic review search strategy (registered with the Center for Open Science (OSF) (DOI 10.17605/OSF.IO/UM95Y).

Co-culture of chondrocytes and mesenchymal stem cells for chondrogenesis in the head and neck

Michael Lee[1,2], Payal Mukherjee[1], Daniel Steffens[1,2], Johnson Chung[3], Gordon Wallace[3]

1. Institute of Academic Surgery – RPA, Sydney, Australia
2. University of Sydney, Sydney, Australia
3. University of Wollongong, Wollongong, Australia

Research Question:

Does co-culturing primary chrondrocytes and mesenchymal stem/stormal cells enhance its ability for chondrogenesis in the head and neck

Searches:

A sensitive electronic search will be performed in MEDLINE via Ovid, Embase via Ovid, BIOSIS Previews using Web of Science using a combination of Medical Subject Headings (MeSH) terms and titles for “Coculture Techniques”, “coculture”, “elastic cartilage” and “chondrogenesis”, “stem cells”, “mesenchymal stromal cells”. In addition, citation tracking of the included studies and relevant systematic reviews will be conducted. No date or language restrictions will be applied.

Initially, one review author will remove clearly irrelevant studies. Screening of titles and abstracts of potentially eligible studies will be performed by two independent review authors. Disagreements over the eligibility of particular studies will be resolved through discussion with a third reviewer.

Table 1. Search strategy	
MEDLINE *via Ovid* (102 results)	
#1	Co-culture.m_titl. OR co-cultures.m_titl. OR coculture.m_titl. OR cocultures.m_titl. OR Coculture Techniques/
#2	Cartilage regeneration.m_titl. OR cartilage generation.m_titl. OR chondrogenesis.m_titl. OR tissue regeneration.m_titl. OR tissue generation.m_titl.
#3	#1 OR #2
#4	Chondrocytes.m_titl.
#5	Stem cells.m_titl. OR mesenchymal stromal cells.m_titl.
#6	#3 AND #4 AND #5
Embase *via Ovid* (161 results)	

#1	Co-culture.m_titl. OR co-cultures.m_titl. OR coculture.m_titl. OR cocultures.m_titl. OR coculture/
#2	Cartilage regeneration.m_titl. OR cartilage generation.m_titl. OR chondrogenesis.m_titl. OR tissue regeneration.m_titl. OR tissue generation.m_titl.
#3	#1 OR #2
#4	Chondrocytes.m_titl.
#5	Stem cells.m_titl. OR mesenchymal stromal cells.m_titl.
#6	#3 AND #4 AND #5
BIOSIS Previews *via Web of Science* (68 results)	
#1	TI=co-culture OR TI=co-cultures OR TI=coculture OR TI=cocultures
#2	TI=cartilage regeneration OR TI=cartilage generation OR TI=chondrogenesis OR TI=tissue regeneration OR TI=tissue generation
#3	#1 OR #2
#4	TI=chondrocytes
#5	TI=stem cells OR TI=mesenchymal stromal cells
#6	#3 AND #4 AND #5
m_titl.=title	

Type of Studies to be Included:

Case reports, case series, in vitro and in vivo studies reporting on the use of chondrocytes with mesenchymal stem/stromal cells in co-culture for tissue engineering

Condition or domain being studied:

Chondrogenesis via co-culture in the head and neck

Intervention(s), exposure(s):

Inclusion criteria:

(i) Study reports on the co-culture of chondrocytes with mesenchymal stem/stromal cells
(ii) Study reports on chondrogenesis in the head and neck

Comparator(s)/control:

Not applicable.

Context:

No restrictions will be placed on the setting or context of the included studies.

Primary outcome(s):

(i) Enhancement of cartilage regeneration

Secondary outcome(s):

None.

Data extraction (selection and coding):

A standardised form will be used to extract data from eligible studies for assessment of the study quality and evidence synthesis. Two independent review authors will extract the data. Disagreements over the data extraction will be resolved through discussion with a third reviewer. Missing information will be requested from study authors via email.

Risk of bias (quality) assessment:

Risk of bias for longitudinal studies will be assessed using the QUIPS tool (Hayden JA, van der Windt DA, Cartwright JL, Côté P, Bombardier C. Assessing bias in studies of prognostic factors. Ann Intern Med. 2013;158(4):280-6).Risk of bias will be rated as “high”, “moderate” or “low” risk according to the following domains: (i) Study participation; (ii) Study attrition; (iii) Prognostic factor measurement; (iv) Outcome measurement; (v) Study confounding; (vi) Statistical analysis and reporting.

Dissemination plan:

The results of this review will be submitted to a peer-reviewed journal for publication as well as presented in national and international conferences.

Keywords:

Co-culture, chondrocytes, mesenchymal stem cells, mesenchymal stromal cells, chondrogenesis, cartilage regeneration

Australian Society of Otolaryngology Head and Neck Surgery 71st Annual Scientific Meeting (17 – 19th September 2021) Poster Presentation

Co-culture of chondrocytes and mesenchymal stem cells for cartilage regeneration in the head and neck

Michael Lee, Payal Mukherjee, Daniel Steffens, Johnson Chung, Gordon Wallace
Institute of Academic Surgery – RPA, New South Wales, Australia

Excellence in ENT

ASOHNS 71ST ANNUAL SCIENTIFIC MEETING

Background:
Cartilage is an ubiquitous tissue in the head and neck with a limited ability for self-regeneration and repair. Congenital cartilagenous abnormalities and acquired deformities secondary to trauma or surgery represent a particular challenge for reconstructive surgery. Conventional techniques such as autologous cartilage reconstruction are efficacious at achieving functional results but are limited by cartilage donor site morbidity and difficulty achieving aesthetic results. The use of non-absorbable implants allows for better aesthetic results but is limited by high rates of implant extrusion and infection.

The use of absorbable implants comprised of bio-degradable scaffolding and cell based "bio-inks" is an emerging technology that seeks to address these challenges. The use of primary chondrocytes as a bio-ink is well established but remains limited by the need for sufficient harvested cartilage to repair large head and neck defects remains limited by donor site morbidity. Mesenchymal stem/stromal cells (MSCs) have been investigated as an alternative bio-ink for chondrogenesis that can be more readily harvested in large quantities, but its use has been limited by the poor quality of formed cartilagenous matrices.

The advantages of primary chondrocytes co-cultured with MSCs for articular cartilage regeneration in degenerative cartilage diseases, such as osteoarthritis, is now widely reported in the literature. The use of co-cultured cells for cartilage regeneration in the head and neck remains an emerging field which will potentially offer novel alternatives to reconstructive surgery.

Method:
A systematic electronic search was conducted through MEDLINE (1946 –), Embase (1947 –) and BIOSIS Previews (1926 –) on May 21st 2021. A search strategy was designed for each database to identify all relevant studies on co-culture. Search terms included co-culture, cartilage regeneration, tissue regeneration, chondrogenesis and stem cells written in or translated to English, published in a peer-reviewed journal and specifically discussing the use of chondrocytes and stem cells in co-culture and their outcomes for chondrogenesis in the head and neck. The primary outcome was degree of chondrogenesis in comparison with mono-culture of chondrocytes or MSCs.

Results:
The search strategy identified 331 articles of which 164 were removed as duplicates. After screening and application of inclusion and exclusion criteria, 30 articles were identified with an additional 5 from through secondary screening. Further screening of these 35 full text articles yielded 15 studies that were included in this review.

Author (Year)	Chondrocyte source	MSCs source	Co-culture ratio	Duration
Acharya (2011)	Human articular Human nasal septal	Human bone marrow Human adipose tissue	1C:3MSC	21 days
Anderson-Baron (2021)	Human nasal septal	Human bone marrow	1C:3MSC	42 days
Apelgren (2017)	Human nasal septal	Human bone marrow	1C:4MSC	60 days
Cai (2014)	Human auricular	Human adipose tissue	3C:7MSC	56 days
Cohen (2018)	Human auricular	Human bone marrow	1C:1MSC	90 days
Goh (2016)	Human auricular	Human adipose tissue	Unspecified	21 days
Kang (2013)	Porcine articular	Porcine bone marrow	1C:1MSC, 2C:3MSC, 3C:7MSC, 1C:4MSC, 1C:9MSC	56 days
Kang (2012)	Porcine auricular	Porcine bone marrow	1C:1MSC, 1C:9MSC	98 days
Lv (2012)	Porcine auricular	Human adipose tissue	3C:7MSC	56 days
Ma (2020)	Porcine auricular	Porcine adipose tissue	1C:1MSC	28 days
Moller (2017)	Human nasal septal	Human bone marow	1C:4MSC	60 days
Morrison (2018)	Porcine auricular Porcine tracheal	Porcine adipose tissue	1C:1MSC, 1C:2MSC 1C:5MSC, 1C:10MSC	56 days
Pleumeekers (2015)	Bovine auricular Bovine nasal septal	Human bone marrow	1C:4MSC	56 days
Zhang (2014)	Human auricular	Caprine bone marrow	1C:3MSC	84 days
Ziegler (2020)	Porcine auricular	Human adipose tissue	1C:5MSC, 1C:9MSC	70 days

Discussion:
Auricular cartilage was the most common source of primary chondrocytes (n=10) followed by nasal septal (n=5), articular (n=2) and tracheal (n=1). Bone marrow was the most common source of MSCs (n=9) followed by adipose tissue (n=7). There was a great variability in technique, with co-culture ratios ranging from 1:1 to 1:10. When compared with equivalent total cell counts between groups, with all techniques, the majority of studies showed the chondrogenic potential of co-cultured cells was superior to mono-culture of MSCs in terms of ECM deposition, chondrogenic gene expression, weight, diameter and height. Co-culture of chondrocytes demonstrated increased elastin/elastic fibre formation than either mono-culture group, a particular important attribute for head and neck reconstruction where the majority of structures consist of elastic cartilage.

Conclusion:
Despite fewer chondrocytes required, the chondrogenic potential of co-cultured cells was similar or superior to mono-culture which supports it's use in head and neck reconstructive surgery where acquiring donor cartilage is a significant limiting factor.

ASOHNS THE AUSTRALIAN SOCIETY OF OTOLARYNGOLOGY HEAD AND NECK SURGERY

Isolation of sheep chondrocytes full protocol

Isolation of Sheep chondrocytes

Chemicals and reagents

- Sterile PBS buffer
- Collagenase type II
- Hams F-12 (Gibco)+ 10% FBS + 1% P/S+2.5ug/ml amphotericin B and 0.1mM ascorbic acid
- Trypan Blue
- Collagenase II [Invirogen-Gibco-1701-015].

Procedure

Washing & dicing

1. Note the age and sex of the sheep
2. Transfer biopsy into a sterile pre-weighed Petri dish.
3. Excise and discard any bone or bloody tissue
4. Weigh the cartilage then add PBS (supplemented with 10vol% pen strep)
5. Dice the sample into approximately 1 mm^3 pieces.
6. Rinse cartilage pieces twice with PBS (supplemented with 10vol% pen strep)
7. Remove PBS, transfer cartilage pieces to a 50ml falcon tube containing digestion media (Hams F-12 + 10 v/v% FBS + 0.15 w/v% collagenase type II)
8. Isolation

To work out digestion solution. We do 10% digestion. Original solution is 1.5%w/v. Final concentration is 0.15% w/v. Weigh cartilage.

If ~2g: Then its 2g+18ml (Media+10% FBS)

X x 1.5% w/v=18ml x 0.15% w/v

V=1.8ml

Thus: 1.8ml collagenase + 16.2 ml media

9. Add collagenase type II Hams F-12 supplemented with 10% FBS (end [collagenase] = 0.15 w/v%) *sterile filtration required if the collagenase is not sterile.

10. Put on an inverter (ensuring proper mixing of the chunks) overnight (5-8h) at 37°C, 5% CO_2. Tape to secure
11. Pipette digest up & down
12. Filter through 100um cell strainer on 50ml falcon tubes
13. Centrifuge at 700g for 5 min, aspirate supernatant off, re-suspend cells in PBS

14. This should be done 3 times
15. Re-suspend cells in chondrocyte medium
16. Count cells using haemocytometer (use trypan blue staining for viability). Note density
17. Seed in T25 flask

Labelling:

- Sheep 1 - Ear 1, Ear 2, Nose
- Sheep 2 - Ear 1, Ear 2, Nose
- Sheep 3- Ear 1, Ear 2, Nose

Isolation of Sheep BM

Chemicals and reagents

- Sterile PBS buffer
- Collagenase type II
- DMEM-LG + 1% glutamax+10% FBS+1%P/S
- Trypan Blue

Procedure

1. Harvest 1-5ml of BM in hyparinised syringe
2. Centrifuge 200g for 6 mins,
3. Centrifuge at 700g for 5 min, aspirate supernatant off, re-suspend cells in PBS
4. This should be done 3 times
5. Culture in DMEM-LG+1% glutamax+10% FBS+1% P/S
6. Plate in T25 or (T75 if there looks like a lot of cells)
7. Remove non-adherent cells after 24 hours. Change media every 3-4 days
8. Stock down

Labelling:

- Sheep 1 - BM1
- Sheep 2 - BM2

Proliferation of sheep chondrocytes full protocol

Cell Proliferation Assay

Procedure

1. 1. Following thawing cells and passaging perform a cell count for total number of live
2. cells
3. 2. Replate cells into 6-well plates at cell densities of 1.5 x10 4 cells/cm 2 (i.e. 1.425 x10 5
4. cells/well).
    a. You will need 2 plates for 6 time points
        i. D1
        ii. D4
        iii. D7
        iv. D10
        v. D12
        vi. D14
    b. You will need to come in on one day over the weekend if starting on a Monday
5. Plate in duplicate
6. Use 1ml of 0.25M Trypsin (Gibco) to detach cells
7. Perform a cell count for each well/time point

Animal ethics approval

THE UNIVERSITY OF SYDNEY

RESEARCH INTEGRITY & ETHICS ADMINISTRATION

Animal Research Authority (ARA)

This ARA must be kept in the facility/farm where animals are housed or carried with you during field work

Animal Research Authority

Project title:	Maxillomandibular Plasma Ion Immersion Implantation Treated Poly Ether Ether Ketone (PEEK) Implant, Scapular Periosteal Bioreactor, Autologous Adipose-derived Stem Cells and Chondrocyte Implantation Project
Project number:	2021/2004
Chief investigator:	Jonathan Clark
ARA approval period:	15 December 2021 – 15 December 2022 An annual report must be submitted and approved prior to the ARA expiration date above.
Project type:	Experimental (non-wildlife)

In compliance with Section 27 of the NSW *Animal Research Act 1985*, this Animal Research Authority (ARA) remains in force for a period of 12 months, unless cancelled sooner.

Renewal of the ARA is conditional upon submission of a satisfactory annual report to the AEC in accordance with the *Australian code for the care and use of animals for scientific purposes 8th Edition 2013*.

Research Integrity & Ethics Administration
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ABN 15 211 513 464
CRICOS 00026A

This ARA must be kept in the facility/farm where animals are housed or carried with you during field work

Wednesday, 15 December 2021

Prof Jonathan Clark
Central Clinical School: Surgery; Faculty of Medicine and Health
The University of Sydney

Email: jonathan.clark@sydney.edu.au

Dear Prof Clark

I am pleased to inform you that the University of Sydney Animal Ethics Committee (AEC) has approved your project entitled **"Maxillomandibular Plasma Ion Immersion Implantation Treated Poly Ether Ether Ketone (PEEK) Implant, Scapular Periosteal Bioreactor, Autologous Adipose-derived Stem Cells and Chondrocyte Implantation Project"**.

Project duration:	15 December 2021 – 15 December 2024 At the end of the project duration (3 years) a completion report must be submitted.
ARA expiry date:	15 December 2022
Authorised investigators:	Clark Jonathan; Burrows Chris; Kolakshyapati Pranish; Li Qing; McKenzie David; Mukherjee Payal; Nessiem Andrew; Williams Zoe; Wise Innes; Zreiqat Hala; Chung Johnson; Crook Jeremy; Liu Xiao; Maruf D S Abdullah Al; Parthasarathi Krishnan; Tomaskovic-Crook Eva; Wallace Gordon;

Project description:
Cancers affecting the head and face require aggressive removal of soft and bony tissue to ensure complete clearance. In minor mineralized defects, bony continuity is restored by the undifferentiated blood cells of the local bone to fill the defect. However, this mechanism is insufficient to fill critical-sized defects of the craniofacial skeleton, which can be reconstructed using prosthetics or grafts to restore form. At present, bony free flaps are commonly used for restoring large jawbone defects; however, donor site morbidity can affect a patient's quality of life, and the reconstructed bone rarely replicates the shape of the bone resected, leading to functional or aesthetic compromises. Biocompatible substitutes for jawbones possessing biomechanical properties more similar to the jawbone, such as Polyetheretherketone (PEEK), could be an alternative to using bony free flaps could also reduce the risk of donor site morbidity and time required for the surgical procedure.

Various potential biomaterials and bone substitutes have been investigated using positive stimulation required for bone regeneration either from bone or periosteum. However, it is still unclear if bone and periosteum promote an equal level of bone growth. In this study, we will use polycaprolactone (PCL), and gelatin methacryloyl (GelMA) hydrogel, bone morphogenetic protein-2 (BMP-2), and tricalcium phosphate (TCP) to regenerate ectopic bone tissue in a bi-layered, in vivo PEEK bioreactor, which basically serves as housing for the contents.

Adipose or fat tissue-derived stem cells (ADSCs) are of particular interest in bone tissue engineering (BTE) because they can differentiate into bone cells. ADSCs have also been used to accelerate critical-sized long bone defect repair in vivo. In this animal study, we will encapsulate ADSCs and BMP-2 in GelMA hydrogel and implant these constructs subcutaneously as well as in the scapula in a sheep model to investigate the bone-forming potential of ADSCs, which can be applied in segmental lower jawbone defect repair in the future. The aim is to investigate how these ADSCs can regenerate bone tissue using the osteogenic potential of periosteum and scapular bone. This will provide strong rationale for the next phase of the study to investigate the potential of ADSCs in a segmental defect repair in mandible.

The outer ear is a highly complex 3D shape comprising mainly of elastic cartilage, which can be deformed by diseases such as microtia (a congenital condition causing malformed ear development) or trauma or destructive diseases such as skin cancer needing segmental resection of the ear cartilage. Current results of reconstructive surgery are poor, need multiple stages, give asymmetric results, and create graft and donor site complications. 3D cartilage bioprinting could address these challenges as it allows us to create customized 3D printed implants using cartilage cells. However, it is unclear what the minimum cell density is required for these cartilage cells to grow into complete cartilage. This study will print primary ovine cartilage cells in various densities alongside PCL and GelMA which will be implanted subcutaneously to determine the appropriate cell density for successful cartilage formation over time.

Documents approved:

Date	Type	Document
25/11/2021	Other	AEC application with track change
27/10/2021	Standard Operating Procedure	SOP anaesthetic recovery
27/10/2021	Standard Operating Procedure	SOP surgical approach
27/10/2021	Monitoring Sheet	Monitoring sheets
27/10/2021	Standard Operating Procedure	SOP-anaesthesia analgesia
27/10/2021	Standard Operating Procedure	SOP husbandry
27/10/2021	Standard Operating Procedure	SOP emergency procedures
27/10/2021	Standard Operating Procedure	SOP husbandry Mcmasters

Animals approved:
Please refer to the document at the end of this letter, which details your approved animal usage and location(s).

Conditions of approval

1. An annual progress report or completion report must be submitted and approved before the anniversary of approval of the project.

2. The following documentation must be kept in the facility where your animals are housed or with you when undertaking fieldwork:
   - A copy of this ARA
   - Emergency contact details in case of an animal emergency
   - Approved monitoring records

3. This project must be conducted according to the application approved by the AEC, including continuing compliance with the conditions outlined in this ARA and with the relevant state/territory animal welfare legislation, the Australian code for the care and use of animals for scientific purposes 8th edition 2013 (the Code), the University of Sydney Animal Ethics Procedures and all other relevant legislation.

4. Animals in captivity must be monitored by approved investigators (or animal care staff under a formal agreement with facility management) at least twice weekly (with even spacing throughout the week). Where applicable, more frequent monitoring must be adhered to in accordance with the approved project. The husbandry and environmental conditions of captive animals must be monitored daily. This may be performed by the facility animal care staff.

5. Any changes to the project must receive written approval from the AEC before they are implemented.

This includes obtaining AEC approval for any changes to experimental procedures, animal numbers, personnel, source of animals or location of animals.

6. All unexpected adverse events that may impact on the wellbeing of an animal must be reported to the AEC within 48 hours, as per Clause 2.1.5 [v] [d] and 2.4.34 [ii] in the Code.

7. Personnel working on this project must be sufficiently qualified for their role by education, training and experience, or adequately supervised.
All new investigators must successfully complete the Introduction to Animal Research (ITAR) course.

8. All animal enclosures (e.g. pens, cages and containers) must be clearly identified with chief investigator name, number of animals, DOB if provided and date of arrival, sex and strain.

9. The AEC must be notified if rodents are required to be singly housed for more than 4 weeks.

10. Research data must be retained and stored in accordance with the relevant legislation and University guidelines.

11. Any drugs to be used for procedures involving animals must be within date (not expired) and stored appropriately as per the manufacturer's recommendations. It is the responsibility of the Chief Investigator to ensure that relevant and current authority for the use of restricted drugs is obtained.

Please contact the Ethics Office at animal.ethics@sydney.edu.au should you require further information.

Yours sincerely

[REDACTION]

Professor Michael D'Occhio
Chair
Animal Ethics Committee
On behalf of the University of Sydney

The University of Sydney AECs are constituted and operate in accordance with the NSW Animal Research Act 1985 and its associated Regulations, the Australian code for the care and use of animals for scientific purposes 8th Edition 2013 and the Australian Code for the Responsible Conduct of Research 2018. All personnel named on the project should be conversant with these documents.

This ARA must be kept in the facility/farm where animals are housed or carried with you during field work

Animals approved:

Country	State	Invasiveness	Location	Classification one	Classification two	Common/strain name	Approved	Total used to date
Australia	NSW	5. Major surgery with recovery	Charles Perkins Centre (D17 McMaster Annex (B02)	Domestic mammals	Sheep	Ovis aries	6	0

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