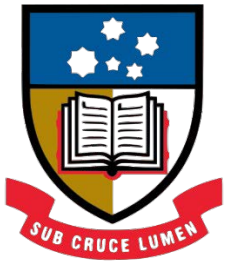

THE UNIVERSITY of ADELAIDE

Osteochondroreticular Stem Cell Therapy for Osteoarthritis: The right cells for the job.

A thesis submitted in fulfilment for degree of
DOCTOR OF PHILOSOPHY

In

School of Medicine
The University of Adelaide

By

Jia Qi Ng

30.10.2019

Declaration

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

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Table of Contents

Thesis Abstract

Osteoarthritis (OA) is a disease of synovial joints characterized by clinical symptoms and pathology related to the whole joint including the periarticular tissues such as the muscles, ligaments and bone. Central to the pathogenesis and diagnosis, however, is the degeneration of articular cartilage. Current treatments include lifestyle modification to prevent further injury, analgesic drugs to help reduce symptoms and surgical interventions such as joint replacement. More recently stem cell therapies, either stimulating endogenous populations or infusing new cells, have been investigated. While OA is a multifactorial disorder, as a foundation to stem cell therapeutics one must accept the premise that OA pathogenesis, at least in part, is contributed to by stem cell dysfunction. Perhaps, a relative insufficiency of developmental articular chondrogenesis, inadequate replenishment during adult homeostasis or incomplete repair following mechanical and inflammatory challenges to cartilaginous integrity such as injury, obesity and ageing. Following the discovery of skeletal stem cells marked by the expression of Gremlin 1 (Grem1) being shown to give rise to chondrocytes in vivo, we studied transgenic mice to trace the Grem1-expressing cells in comparison to other MSC populations marked by the expression of Leptin receptor (LepR) and a common chondrocyte marker Aggrecan (Acan) in the knee articular cartilage. We found that Grem1-expressing cells label a stem cell population within the mouse knee articular cartilage in development as well as in adulthood. We then used a mouse model of OA, destabilisation of the medial meniscotibial (DMM) surgery, in these transgenic mice to test how injury modulates this population. We evaluated the single cell expression of these cells in the knee articular cartilage, by scRNASeq and generated a new mouse line to allow easy ablation of the Grem1 expressing cells within the knee articular cartilage. We also expanded these cells in vitro both to study their relative in vitro characteristics, as well as for injection back into our OA disease model. This thesis presents a comprehensive re-evaluation of articular stem cell biology and applies it to the important and increasingly global clinical problem of osteoarthritis. This work has made new discoveries, generated new transgenic mouse reagents and has stimulated ongoing translational research to better understand osteoarthritis and develop new approaches to its prevention and treatment.

Table of Abbreviations

5-Ethynyl-2'-deoxyuridine	EdU	Human leukocyte antigen	HLA
Aggrecan	Acan	In situ hybridization	ISH
Alpha-smooth muscle actin	αSMA	Induced Pluripotent stem cell	iPSC
Articular cartilage stem-progenitor cell	ACSC	Inducible diphtheria toxin receptor	iDTR
Australia/New Zealand	ANZ	Interleukin 1 beta	IL-1β
Autologous chondrocyte implantation	ACI	Interleukin 6	IL-6
Bone marrow mesenchymal stem cell	BM-MSC	Interleukin 17	IL-17
Bone morphogenetic protein	BMP	Intraperitoneal	IP
Cathelicidin	Camp	Leptin Receptor	LepR
Clustered regularly interspaced short palindromic repeats	CRISPR	Matrix metalloproteinase	MMP
		Matrix-induced autologous chondrocyte implantation	MACI
Colony forming-unit fibroblasts	CFU-F		
Collagen type I alpha 2	Col1a2	Mesenchymal stem cell	MSC
Collagen X	ColX	Nestin	Nes
Complementary DNA	cDNA	Osteoarthritis	OA
Decorin	DCN	Osteocalcin	OCN
Destabilisation of the medial meniscotibial	DMM	Osteochondroreticular	OCR
		Parathyroid hormone-related protein	PTHrP
Dickkopf-related protein 1	DKK-1	Proliferating cell nuclear antigen	PCNA
Diphtheria toxin	DT	Proteoglycan 4/ Lubricin	Prg4
Diphtheria toxin fragment A	DTA	S100 calcium-binding protein A9	S100a9
Down-regulated in mos-transformed cells	Drm	Single-cell RNA sequencing	scRNA
	ECM	Thrombospodin motifs	ADAMTS
Extracellular matrix	FRP	Tumour growth factor-beta	TGF-β
Frizzled-related protein	Grem1	Tumour necrosis factor	TNF
Gremlin 1	Gdf5		
Growth and differentiation factor 5	HSC		
Haematopoietic stem cell			

Publications Arising From This Thesis

Jia Ng, Christopher B. Little, Susan Woods, Samuel Whittle, Francis Y. Lee, Siddhartha Mukherjee, David J. Hunter, Daniel L. Worthley. Stem Cell Directed Therapies for Osteoarthritis: the promise and the practice (Review). **Stem cells, 2019**

*Denotes equal primary contributors to publication

^Denotes accepted for publication however not currently in press

Additional Publications Arising From Outside This Thesis

During my PhD candidature, I was involved in several other published studies as both primary and co-author/investigator. These are as follows:

Lannagan RM, Lee YK, Wang T, Roper J, Bettington ML, Fennell L, Vrbanac L, Jonavicius L, Somashekar R, Gieniec K, Yang M, **Ng JQ**, Suzuki N, Ichinose M, Wright JA, Kobayashi H, Putoczki TL, Hayakawa Y, Leedham S, Abud HE, Yilmaz OH, Marker J, Klebe S, Wirapati P, Mukherjee S, Tejpar S, Leggett BA, Whitehall VLJ, Worthley DL, Woods SL. Genetic editing of colonic organoids provides a molecularly distinct and orthotopic preclinical model of serrated carcinogenesis. ***Gut, 2018 Apr 17.***

*Denotes equal primary contributors to publication

^Denotes accepted for publication however not currently in press

Chapter 1: Stem Cell Directed Therapies for Osteoarthritis: the promise and the practice

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Samuel Whittle: Conception and design, manuscript writing

Francis Lee: Conception and design, manuscript writing

Stan Gronthos: Conception and design, manuscript writing

Siddhartha Mukherjee: Conception and design, manuscript writing

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MeSH terms: stem cells, OA, chondrocytes, articular cartilage.

1.4 Abstract

Osteoarthritis (OA) is a disease of an entire synovial joint characterized by clinical symptoms and distortion of joint tissues including cartilage, muscles, ligaments and bone. While OA is a disease of all joint tissues it is a defined accessible compartment and is thus amenable to topical surgical and regenerative therapies, including stem cells. All tissues arise from stem progenitor cells, and the relative capacity of different cellular compartments, and different individuals, to renew tissues into adulthood may be important in the onset of many different degenerative diseases. OA is driven by both mechanical and inflammatory factors, but how these impacts the proliferation and differentiation of cells into cartilage in vivo is largely unknown. Indeed, our very basic understanding of the physiological cellular kinetics and biology of the stem-progenitor cell unit of the articular cartilage, and how this is influenced by mechanoinflammatory injury, is largely unknown. OA seems, rather deceptively, to be the low-hanging fruit for stem cell therapy. Without the basic understanding of the stem cell and

progenitor unit that generate and maintain articular cartilage in vivo, we will continue to waste opportunities to both prevent and manage this disease. In this review, we discuss the biology of chondrogenesis, the stem cell populations that support articular cartilage in health and disease and future opportunities afforded through the translation of basic articular chondrocyte stem cell biology into new clinical therapies.

1.5 Statement of Authorship

Statement of Authorship

Title of Paper	Contribution of Gremlin 1 expressing stem cells to cellular architecture and stem cell biology of articular cartilage in development and adulthood.
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Principal Author

Name of Principal Author (Candidate)	Jia Ng		
Contribution to the Paper	First author and main contributor. Concept design, literature search, review and formulation of primary draft, in addition to reviewing and incorporating co-author comments and suggestions.		
Overall percentage (%)	90%		
Certification:	I am the primary author of this paper.		
Signature		Date	30/10/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Christopher Little		
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1.5 Introduction

1.5.1 Osteoarthritis (OA)

Osteoarthritis (OA) is an expensive, common and debilitating condition. Globally, OA is estimated to affect more than 220 million people. The cost of OA in the USA, Canada, UK, France and Australia is estimated to account for between 1% and 2.5% of their respective gross national product[1]. The burden of OA on our people and our pockets is expected to increase exponentially in the coming decades as the population continues to age and the obesity rate rises.

OA seems a promising candidate for regenerative stem cell therapy. OA is a disease of all of the joint tissues (i.e. the whole joint organ), albeit the mesenchymal components of the joint are amenable to both local and systemic therapy. But, the multiple distinct tissues cartilage, ligament, tendon, bone, muscle, synovium, capsule, raise the immediate issue that for stem cell therapies to be effective either one cell must differentiate into and migrate appropriately to regenerate all joint tissues, or what is perhaps more likely, is the separate pools of adult stem cells may need to be modulated, replaced, regenerated to specifically address discrete joint deficiencies.

Articular cartilage is a prominent contributor to OA pathology and has been the focus of much research. Our understanding of adult mesenchymal stem cells (MSCs), and their easy propagation and differentiation into chondrogenic cells in vitro has skewed the field's general thinking that all cartilage is the same, or at least a cell that can form cartilage in vitro can form and is the origin of, all cartilage in vivo. But, therein lies the issue, all cartilage is not the same and in vitro behaviour may not reflect in vivo cell biology particular in the setting of disease.

As we will learn below articular cartilage is a distinct compartment, that performs discrete functions and is susceptible to discrete insults. Importantly, it is likely that articular cartilage and other cartilaginous compartments are maintained by different stem-progenitor pools to the rest of the skeleton. This may explain why, despite our high hopes, stem cell therapy has, so far, been relatively disappointing in treating OA. We need to better understand the articular cartilage compartment and the endogeneous stem cell hierarchy, in order to better treat our patients. In the following review, we will examine our current understanding of skeletal stem cells, cartilage development and biology, and the practice and promise of stem cell therapies for OA.

1.5.2 Developmental biology of bone and articular cartilage

The prevailing model for the development, growth and repair of long bones occurs in two phases, known as endochondral ossification[2]. First, cartilage cells and surrounding matrix form a "scaffold" for bone formation. Osteoblasts then invade this matrix and lay down the mineralized parts of bone[2]. Although this process has been known for decades, it has perhaps been used inappropriately as an analogy for all circumstances of skeletal mesenchymal regeneration. Furthermore, from several studies, it appears that primitive chondrocytes do not serve merely as a transient template but can also differentiate into mature bone and cartilage as well as associated stromal reticular cells[3-4]. As the bone elongates, haematopoietic stem cells (HSCs) relocate from the liver to the bone marrow, establishing a perisinusoidal niche with a population of supportive adult MSCs[2]. With increasing ossification in the secondary ossification centers within the epiphyses, the articular cartilage is increasingly remote from the bone marrow proper, squeezed between the synovial fluid and the advancing subchondral bone. This establishes a remote and very specific compartment, the articular compartment, forged in

unique circumstances for a unique function. The articular cartilage deserves special attention, particularly when searching for new cellular therapies for OA.

1.5.3 Articular cartilage stem cell biology

Stem cell compartments are characterised by their functional and structural organisation, their self-renewing zone, their progenitor zone, their subsequent differentiation and the various lineages that are formed (**Table 1**). Cells with greater self-renewal and multipotency give rise to cells that progressively lose these faculties, influenced by discrete autocrine and juxtracrine signalling.

Cell type	**Identifying markers**	**Location**	**In vivo contributions**
BM-MSC	Nes/ LepR (mouse)	Bone Marrow – Perisinusoidal	Osteocytes, adipocytes, and perivascular cells
BM-MSC	CD146/MCAM (Human)	Bone Marrow – Perisinusoidal	Osteocytes, adipocytes and perivascular cells
Osteochondroreticular (OCR) stem cells	Grem1	Trabecular bone and growth plate	Osteocytes, chondrocytes and reticular cells
Mouse skeletal stem cell (mSSC)	$CD45^{-}Ter\text{-}119^{-}tie2^{-}AlphaV^{+}thy^{+}6C3^{-}CD105^{-}$	Trabecular bone and growth plate	Osteocytes, chondrocytes and reticular cells
Synovium MSC	Gdf5	Joint interzone	Articular cartilage, menisci ligaments, synovium and fat pad
Articular cartilage progenitor cells	Prg4	Embryonic joint surface, Adult articular cartilage	Articular cartilage, synovial cells, tendon and ligament
Articular cartilage stem-progenitor cell (ACSC)	Unknown	Superficial zone of articular cartilage	Articular chondrocytes

Table 1. Summary of the current existing skeletal stem cells.

The MSC model argues that a self-renewing stem cell exists within the bone marrow that gives rise to all mature osteoblasts, chondrocytes, adipocytes, and marrow stromal cells required for skeletal development, homeostasis, repair and HSC support[5]. The endogenous MSC was, at one time, believed to be the reticular mesenchymal cells that surround the bone marrow sinusoids[6]. Perisinusoidal mesenchymal cells are marked by nestin (Nes)-GFP[7] and leptin receptor (LepR)-cre[8, 4, 9] in mice and by CD146/MCAM in humans[10]. Our understanding of MSCs has been both facilitated, but also potentially hampered, by the ease of propagation of such cells *in vitro*. It was believed that *in vitro* multipotency and self-renewal would perfectly reflect *in vivo* behaviour, and thus this cell would explain the origin of all bone and cartilage, the perfect candidate to generate and repair articular cartilage.

Perisinusoidal mesenchymal cells expressing LepR do indeed include multipotent, colony-forming-unit fibroblasts (CFU-Fs)[8]. Furthermore, lineage-tracing studies revealed perisinusoidal population contained cells with *in vivo* osteogenic and adipogenic potential; however, these cells gave rise to osteo-adipogenic lineages exclusively in adult mice (>8 weeks of age) and not during development or bone growth[8, 4, 9]. Furthermore, LepR-expressing perisinusoidal cells do not routinely contribute to normal developing chondrocytes, the major cell lineage generating the cartilaginous matrix required for endochondral ossification[8, 4, 9]. Together, these data raised the prospect that other complementary postnatal skeletal stem cells exist. Importantly the perisinusoidal MSCs did not generate the articular cartilage. The articular cartilage is discrete from the perisinusoidal MSC compartment, so we needed to look elsewhere. that this

Two overlapping skeletal stem cells in mice were subsequently reported, one defined by lineage tracing and expression of the BMP-antagonist *Gremlin1*[3], and the second proven

through cell surface markers selection of $CD45^{-}Ter\text{-}119^{-}tie2^{-}AlphaV^{+}thy^{+}6C3^{-}CD105^{-}$ and transplantation studies[11]. Both satisfied the criteria of a stem cell, self-renewal and the development of multiple lineages, but were distinct from typical MSCs. Interestingly, the *Grem1*+ stem cells had no adipogenic potential and were named the osteochondroreticular (OCR) stem cells to reflect their distinction from traditional MSCs and in reference to the earlier concept of the osteochondroprogenitor[12]. Both the Chan immunophenotyping paper[11] and the OCR paper[3] found that these stem cells were concentrated within the trabecular bone area, consistent with their contribution to the epiphyseal plate and post-natal endochondral ossification. Grem1 is a BMP-antagonist that is highly involved in bone and cartilage regulation. Multiple studies have shown a significant increase in Grem1 expression in human OA samples and surgically induced dog models of OA[13-14]. More recently, Grem1 has been shown to modulate osteoarthritis through the NF-κB pathway[15]. This suggests that perhaps OCR stem cells may also be involved in OA disease progression, but also that the cells, and the genes that the cells express, may have different effects on different regenero-mechano-inflammatory pathways within the whole joint and in the pathogenesis of OA.

These previous studies, however, still did not address the specific organization of articular cartilage, which is anatomically, morphologically and functionally different from the growth plate cartilage, fracture callus and other skeletal tissues. Understanding stem cell dynamics begins with understanding cellular renewal and proliferation. Articular cartilage, as opposed to some other forms of cartilage, is comprised of organised stratified chondrocytes on the joint surface of the bone, all of which are surrounded by a rich extracellular matrix. Chondrocytes are specialised mesenchymal cells, present at many different sites, performing many different functions throughout life. For instance, they are essential for the development of long bones through endochondral ossification and reside there, but then aggregate within the postnatal

bone primarily within the growth plate, the conveyer belt to enable further post-natal long bone growth and at the articulating surfaces of those bones, they help to enable synovial joint function. These chondrocytes within the growth plate are believed to be maintained by resident stem cells that are located in the resting zone of the growth plate and trabecular bone area more generally[16]. Chondrocytes are also found within fracture callus[3], tendon and ligament[17], following bone or ligament injury, where they are believed to develop from local mesenchymal stem-progenitor cells and, as in development, provide a template for bony organisation.

The articular chondrocytes are uniquely arranged into specialised subsets of cartilage cells, organised into morphological zones, named the superficial zone, the middle zone, the deep zone and the calcified zone (**Figure 1**)[18]. It is postulated that the articular cartilage stem cells originate from the joint interzone during embryonic life. Using lineage tracing in mice, researchers uncovered these mesenchymal stem cells marked by the expression of growth and differentiation factor 5 (Gdf5) in the interzone. These Gdf5 cells not only gave rise to the articular cartilage but also new cartilage after injury in adulthood[19].

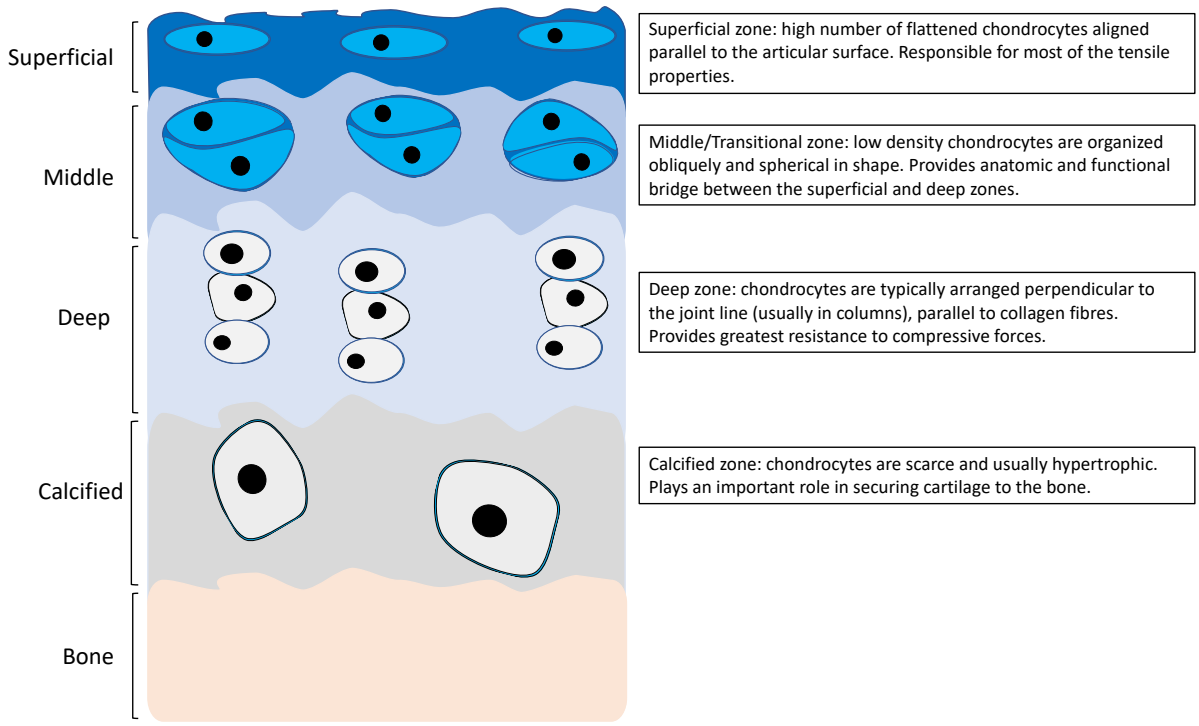

Figure1. Schematic diagram of the different cartilage zones.

Studies using intra-articular BrdU incorporation[20] and *in vivo* genetic[21] approaches, found that the articular cartilage zones are renewed from the superficial zone. That is, cells within the first few layers of the articular cartilage, bathed in synovial fluid, slowly divide, and differentiate into more superficial zone chondrocytes and ultimately the deeper zone chondrocytes as well. This was, in turn, supported by lineage tracing studies using the major articular cartilage lubricant marker proteoglycan 4 (*Prg4*), which showed that the initial labelling in superficial zone chondrocytes, particularly in young mice, slowly gave rise horizontally to other superficial zone chondrocytes and, over time both deeper zone chondrocytes and periarticular bone[22]. Thus, the superficial zone is likely to contain an articular cartilage stem-progenitor cell (ACSC)[23-24], that is distinct in origin, markers, kinetics and differentiation compared to other cartilage progenitors. How this ACSC is best defined, characterized and most importantly how these cells relate to OA, however, is unknown. Although study has suggested that these ASCS can be isolated based on fibronectin adhesion and retains their stemness and articular cartilage

characteristics up to 60 population doubling[25]. The superficial location of the ACSC raises the prospect that OA, characterized by the early damage of superficial chondrocytes may, in part, be a disease of stem cell loss. ACSC apoptosis, exhaustion, and differentiation may compound the vicious cycle of mechanical stress, inflammation and worsening joint function. Thus, rather than forming competing models of OA, stem cell failure may be inherently linked within a more integrated model of mechanical, inflammatory and regenerative perturbation driving the pathogenesis of OA.

1.5.4 Pathogenesis of osteoarthritis

OA is a joint disorder identified by extracellular matrix degradation initiated by abnormal joint tissue metabolism followed by anatomic, and/or physiologic derangements[26]. It is the end result of a combination of genetic, metabolic, biochemical, inflammatory and mechanical predispositions and insults (**Figure 2**), but the loss of articular cartilage is certainly a consequence of this[27]. Ageing, injury and predisposing risk factors such as obesity or genetic risk conspire to initiate OA. The loss of articular cartilage can, in turn, aggravate inflammation, joint misalignment and bony remodelling (subchondral bone destruction and osteophyte development), loss of muscular and ligamentous joint support, and ultimately the defining clinical symptoms of joint pain, instability and stiffness[28].

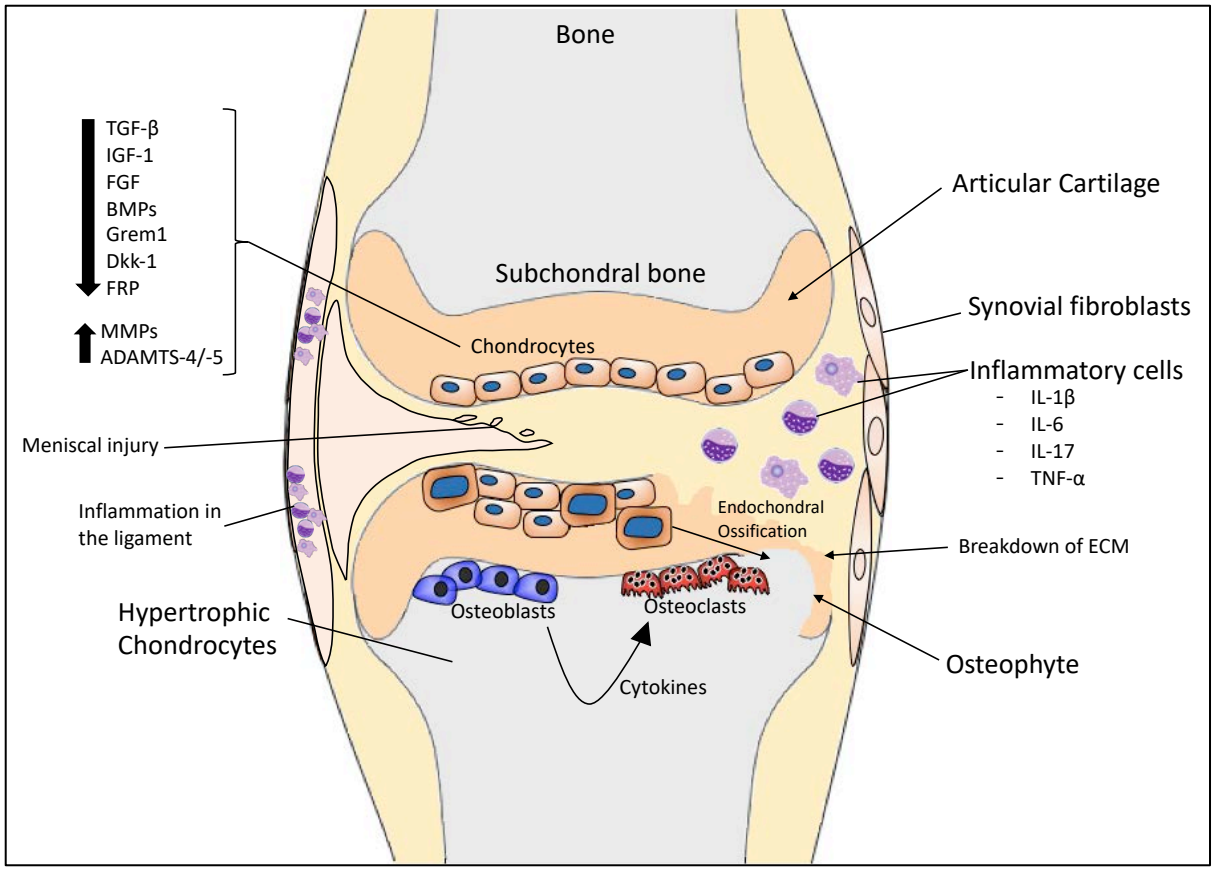

Figure 2. Schematic diagram of OA pathology.

The primary function of articular cartilage is to allow for smooth gliding and protection of the bones from mechanical stresses inherent to synovial joint function[29]. The articular cartilage and the cells that support it are lost in OA. Through a stem cell prism, it could be postulated that articular cartilage and the stem-progenitor cells that give rise to it, may predispose to OA through one of three potentially overlapping, processes: (1) inadequate development – that might be prone to future injury; (2) impaired maintenance of adult articular cartilage contributing to disease; and/ or (3) inadequate repair, following more significant disease. We do not propose that OA is so simple, so unidimensional, but a reductionist approach can help to understand the condition and then examine how stem cell biology interacts with the other mechanical, endocrine, inflammatory and mechanical drivers of disease.

Chondrocytes are sensitive to physical injury, and extreme mechanical forces alter the chondrocyte balance of anabolic and catabolic factors, compounding injury and inducing inflammation[30]. Catabolic enzymes, such as matrix metalloproteinase-13 (MMP-13) the dominant factor in collagen type II degradation, and disintegrin and metalloproteinases with the thrombospodin motifs (ADAMTS)-4 and -5 that degrade the predominant proteoglycan aggrecan are upregulated in the chondrocytes and synovial cells during OA[31,32]. The degraded cartilage fragments are released into the joint and come in contact with the synovium, activating expression and synthesis of inflammatory mediators like interleukin-1-beta (IL-1β), interleukin-6 (IL-6), interleukin-17 (IL-17) and tumour necrosis factor (TNF)[33]. Understanding the complex cellular processes that regulate the physiological and pathological functions of chondrocytes is essential to the development of more effective strategies in treating OA. But, given that the ACSC are within the superficial zone, perhaps the very stem-progenitor cells that should renew the articular cartilage are some of the earliest casualties in OA? Alternatively, does their relative loss establish the necessary preconditions for OA? These questions require of careful characterization of the ACSC in both ageing and OA mouse models as well as marrying these findings to human cells and human disease.

1.6 Current treatment of OA

1.6.1 Lifestyle, pharmaceutical drugs and joint replacement surgery

Currently available strategies for OA focus on prevention, pain relief, improving function and reducing further deterioration[34]. The usual approach includes modifying lifestyle factors (weight loss, exercise and self-management) integrated with pharmaceutical approaches centred on analgesia including non-steroid anti-inflammatory agents, and, where necessary, surgery including joint arthroplasty[35]. Current OA drugs have been associated with side effects and joint replacement surgeries are invasive and costly. Some biological OA therapies have been investigated, including drugs that promote chondrogenesis[36] and osteogenesis, matrix degradation inhibitors, apoptosis inhibitors, and anti-inflammatory cytokines[37]. Importantly, however, none have yet demonstrated a sufficient improvement in symptoms to be included in standard care[38].

1.6.2 Existing Cell therapies

The inadequacy of traditional OA drug treatments has shifted efforts, as in many medical conditions, towards cell-based therapies. Loss of articular cartilage is a central feature of OA and it occurs within a compartment that is immunotolerant and accessible for cell delivery.

Proof-of-concept that cell-based therapy can regenerate articular cartilage has been shown by Brittberg *et al* in the 1990s using autologous chondrocyte implantation (ACI)[39]. ACI requires the extraction of chondrocytes from the non-weight bearing part of the intact joint regions, expanded in culture and transplanted into focal defects in the affected joint[40]. Though tissue engineering efforts such as ACI or matrix-induced ACI (MACI) treatment has been used for

treating osteochondral lesions in patients with concomitant OA, the benefits have been modest, perhaps due to the loss of chondrogenic capacity following *in vitro* expansion along with the difficulties of sustained engraftment, and rapid loss of any progenitors that engraft within the superficial zone due to ongoing injury[41-42]. Thus, from autologous chondrocytes, cell therapy shifted to using readily available, highly proliferative and multipotent MSCs. MSC infusion through direct intra-articular injection and osteochondral grafts accompanied by scaffolds or matrices, have been disappointing in clinical trials, albeit there are ongoing trials to assess adjuvant growth factors and new scaffolding technologies. Microfracture is another approach whereby direct surgical injuring of the articular surface stimulates a predominant fibrovascular inflammatory response, mobilising endogenous mesenchymal progenitors.

Country	Condition	Intervention	Identifier No	Phase	Outcome
USA	Knee OA	Umbilical-cord MSCs	NCT03166865	I and II	Ongoing
			NCT02580695	I and II	Not available
		Wharton Jelly derived MSCs	NCT02963727	I	Ongoing
		Placenta MSCs	NCT03028428	II	Ongoing
		Mesenchymal progenitor cells	NCT02641860	I	PMID: 31639063
		Adipose MSCs	NCT02966951	I	Ongoing
			NCT03000712	NA	Ongoing
			NCT03509025	II	Ongoing
			NCT02855073	II	Ongoing
			NCT02674399	II	Not available
			NCT03379168	NA	Ongoing
			NCT03467919	III	Ongoing
			NCT02838069	II	Not available
		Bone marrow MSCs	NCT02365142	I and II	Not available
			NCT02351011	I and II	Not available
	Knee/Hip OA		NCT03067870	I	Ongoing
Europe	Knee OA	Adipose MSCs	2015-002125-19	IIb	Unknown
Australia and New Zealand	Knee OA	Adipose MSCs	ACTRN12617001095358p	I	Ongoing
	RA/OA		ACTRN12617000638336	NA	Ongoing

Table 2. Summary of the ongoing clinical trials in US, Europe and ANZ.

As of January 2018, a total of 72 clinical trials were registered in the last 10 years in the US, Europe and Australia/New Zealand (ANZ) using MSCs as a treatment of OA. 25 of them currently ongoing as summarised in **Table 2**.

Another recent systematic review summaries the findings of the all clinical stem cell therapy up to 2017 to treat OA has concluded that although the majority of studies have reported symptomatic and radiological improvements, these therapies lack consistency and have conflicting evidence on the long-term maintenance of positive results[43]. We are currently undertaking a Cochrane living systematic review of stem cell injections for osteoarthritis of the

knee that will continuously synthesise the results of existing and ongoing randomised controlled trials of stem cell therapies[44]. Furthermore, the FDA has recently limited medical retailers from offering unproven and potentially harmful stem cell therapies for OA.

One could possibly explain the lack of efficacy because of the enduring injury that prevents stem cell engraftment and repair. Furthermore, the discrepancy in tissue source for MSC isolation, *in vitro* expansion and differentiation to maintain their "stemness", and route of delivery with or without a diversity of carriers has hampered the effectiveness of stem cell treatment for OA[45]. The pitfalls of the existing therapies warrant a continued search for potential new treatments, informed by underlying articular stem cell biology. Many of these temporising procedures have also been shown to be no better than placebo[46] and have failed to provide long-term sustainable treatment[47-48]. Despite the large amount of new information gleaned from *in vitro* and *in vivo* studies, challenges remain in reconciling these heterogeneous studies as well as modifying the complex processes involved in OA pathogenesis in the first place. This is a dilemma common to other stem cell therapies: one can generate the dopaminergic neurons in Parkinson's[49] or the beta cell in Type 1 diabetes mellitus[50], but how to protect these cells from the initial injury that caused the disease in the first place?

1.7 MSCs, ASCS, iPS cells: what is the best practical stem cell population to study in OA regeneration?

1.7.1 Induced Pluripotent Stem Cells (iPSCs)

iPSCs have been widely used in regenerative medicine and drug discovery through characterizing the *in vitro* phenotype of disease-relevant cells from patients[51]. iPSCs are capable of indefinite proliferation without undergoing differentiation but can be induced to become the desirable cell types including chondrocytes. Efficacy and safety of iPSC-derived stem cell therapy remains to be investigated for OA though their therapeutic potential has been tested in other diseaeses[52].

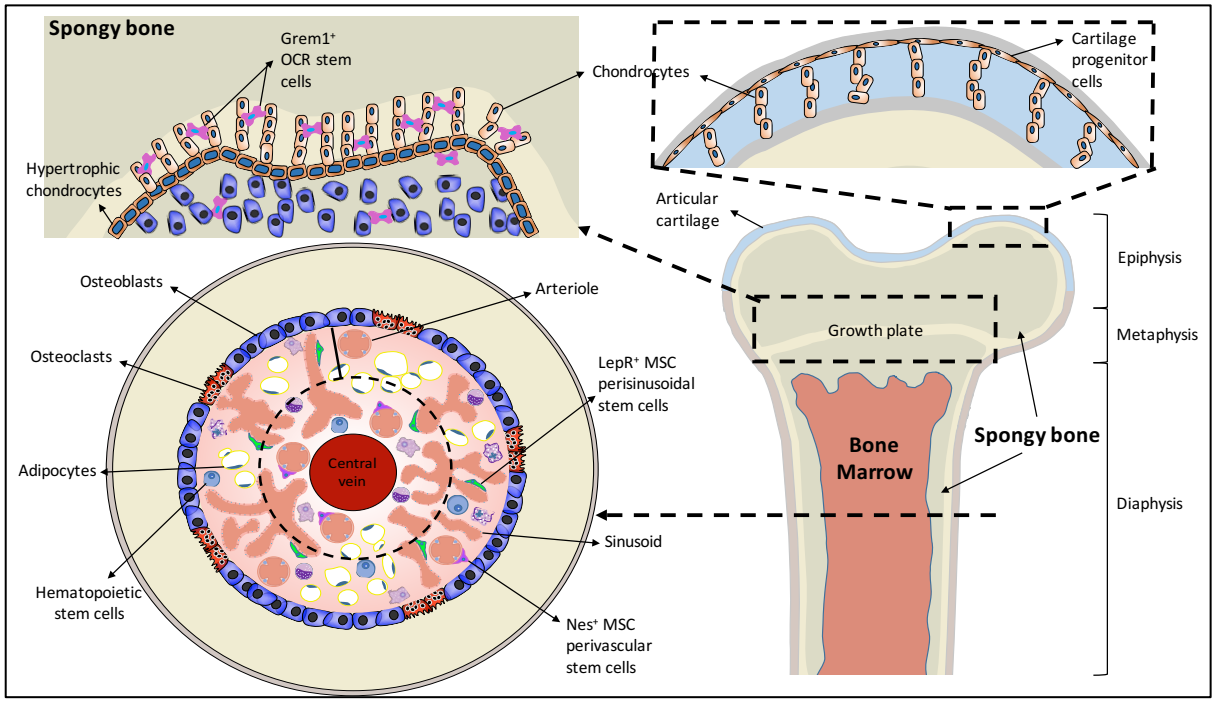

Figure 3. Diagram showing the existence of different type of stem cells with chondrogenic capabilities.

1.7.2 Traditional Mesenchymal Stem cell

There are multiple types of stem cell that exist (**Figure 3**). Adult mesenchymal stem cells (MSCs) can be isolated from bone marrow and other tissues[6] and are expandable through standard culture techniques with reasonable simplicity. They are characterised by their fibroblastic shape, immunophenotype ($CD11b^-$, $CD14^-$, $CD34^-$, $CD45^-$, HLA-DR^-, $CD73^+$, $CD90^+$, $CD105^+$), tri-lineage differentiation potential towards bone, cartilage and adipose tissue, and long-term self-renewal capabilities[53]. MSC contribution to tissue repair and regeneration has been widely documented in skeletal and dental structures[54-55]. More interestingly, they have been shown to further exert their therapeutic efficiency via other immunomodulatory mechanisms due to the lack of human leukocyte antigen (HLA) class II expression. It has also been proposed that stem cells immunomodulatory properties in combination with their paracrine activity and secretion of bioactive molecules, hold a pivotal role in stem cells regenerative capacity[56]. Their immunosuppressive function together with their regenerative capability makes them an attractive candidate for stem cell-based therapy of multifactorial and multi-tissue disease such as OA.

MSC-based regenerative therapy has been widely cited in many preclinical models of OA. Regenerative potential of MSCs are mostly executed in combination with appropriate scaffold materials and chondrogenic induction factors [57-60]. The first stem cell therapy using autologous bone marrow mesenchymal stem cells (BM-MSCs) expanded *ex vivo* for OA treatment has been investigated in humans in 2002[61]. Although arthroscopic and histological improvements were observed in this study, there were no significant difference in clinical results reported[61].

The inconclusive results from these studies was attributed to the cellular heterogeneity of isolated and *in vitro* expanded MSCs from multiple tissue sources[62].

The difficulties in applying stem cell therapy to OA may relate to the cells selected, their means of *ex vivo* expansion/differentiation, how they are administered, the enduring injury that prevents their engraftment and repair, or indeed a combination of these factors. Furthermore, recent advances in stem cell research has suggested that MSC may not be the true endogenous origin of developing bone and cartilage. Contradictory to their *in vitro* lineage repertoire, MSCs do not generate normal articular cartilage or contribute to normal osteochondral skeletogenesis[3, 8, 4, 9].

1.7.3 Different populations of skeletal stem/progenitor cell

We described multiple different adult skeletal stem cell populations above, so which should be used for *in vivo* cartilage regeneration? The short answer is that this remains to be tested, including the value of the varying populations described in recent mouse studies[3, 11, 8]. Some groups have discovered the existence of progenitor cells from the surface zone of both bovine and human articular cartilage, analogous to the mouse ACSCs, described above. These progenitor cells[63] share similar *in vitro* characteristics as the adult bone marrow MSCs. ACSCs have been shown to migrate towards degenerated cartilage sites in late-stage OA[64] and can be mobilised towards injured site via blunt impact or mild enzymatic insult to the ECM in healthy cartilage explants[65]. Although the existence of more than one skeletal stem/progenitor cell population during early embryonic development is evident, identification of such population remains elusive due to the lack of well-defined markers that are necessary for the purification of these cells. Phenotypic change of such cells upon isolation and monolayer expansion in chondrocytes verified the complication in identifying cartilage stem cells[66]. In light of this, it is fundamental to utilise new techniques to identify regenerative cells with not only enhanced

chondrogenic potential but also immunomodulatory function. Perhaps even using multiple discrete skeletal stem cells, each providing unique, essential, properties.

1.8 Other approach to stem cell therapy for OA

An alternative, or indeed complementary approach to cellular replacement is regulating endogenous chondrogenesis within resident or replaced cell populations. The exact mechanisms that govern the regeneration of articular cartilage remain uncertain. Matrix-guided application of stem cell therapy offers delivery of regional growth factors for stem cell regulation. Collagen type I hydrogel is most commonly used as a scaffold for stem cell tissue engineering in cartilage defects. It mimics the material properties of hyaline cartilage and can be metabolised by stem cells through endogenous collagenases to prevent inflammatory rejections[67]. It has also shown hyaline-like cartilage tissue regeneration and significantly improved clinical outcomes up to 5 years in human cartilage defect[68]. Other approaches include the addition of growth factors to enhance stem cell chondrogenesis. Amongst the more extensively examined growth factors are the fibroblastic growth factors and their receptors[69] in particular Fgf18[70] and Vegf[11], TGF-β1, TGF-β3 and BMPs[71]. More recently, it was suggested that the articular cartilage secretes factors such as Gremlin 1 (*GREM1*), Frizzled-related protein (*FRP*) and Dickkopf-related protein 1 (*DKK-1*) that can prevent hypertrophic differentiation[72].

Extracellular vesicle (EV) is a collective term for particles such as exosomes, microparticles and apoptotic classified according to their size and origin[73]. Although they are widely explored as anti-tumour therapy, pathogen vaccination, immunomodulatory and regenerative therapies and drug delivery[74], investigations into EV as a novel therapeutic option for OA has only recently been investigated. Studies in small animal OA models showed that stem cell-derived EV attenuate OA disease progression in vitro and in vivo by displaying similar biological

functions as stem cells[75-76]. The relatively small number of studies have shown the potential for stem cell-derived EV for the treatment of OA, however, many fundamental questions such as mechanisms, kinetics and influence on other cell types within the joint compartment remains to be answered[77].

1.9 Summary

The need for a sustainable long-term effective treatment for OA remains a critical unmet medical need, that continues to grow in size and socio-economic burden with increased ageing and obesity. With the recent discovery of new populations of stem cells with greater chondrogenic potential and the advances in the field of tissue engineering, the clinical use of stem cells as a therapeutic commodity will continue to be studied in our laboratories and clinics. We must learn more about the intrinsic stem cell origin, maintenance and regulation within the articular cartilage, as well as within other stem cell pools within the entire joint organ. Ultimately, integrated scaffolds, with precisely chosen ACSC populations impregnated with discrete and regional growth factors and anti-inflammatory agents may be required to achieve long-term engraftment and improved clinical outcomes. These are exciting times for stem cell biology in the understanding and management of OA.

1.10 Thesis Hypothesis and Aims

Hypothesis – Grem1-expressing OCR stem cells with an enhanced chondrocytic potential may be a new stem cell-based therapeutic option for cartilage diseases. Studies were carried out with lineage tracing mice marking Grem1-expressing population of cells. The studies are compared to traditional MSC in lineage tracing mice marked by the expression of LepR as well as lineage tracing mice marking Acan-expressing chondrocytes. Stem cell properties and contribution to the articular cartilage structure will be compared to the LepR-expressing cells and Acan-expressing cells in vitro and in vivo.

Aims:

To characterise and determine Grem1-expressing OCR stem cells in the articular cartilage and their contribution to articular cartilage structure in postnatal development.

To determine the role of Grem1-expressing articular cartilage stem cells in OA.

To validate the role of Grem1-expressing articular cartilage stem cells in the progression of OA pathology.

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Chapter 2: Contribution of Gremlin 1 expressing stem cells to cellular architecture and stem cell biology of articular cartilage in development and adulthood.

Jia Q. Ng, Yan Ma, Mari Suzuki, David Haynes, Danijela Menicanin, Daniel Worthley

2.1 Abstract

Understanding the cellular architecture and stem cell biology of articular cartilage in postnatal development has always been elusive due to the lack of cellular markers in identifying these cells. Articular cartilage is a highly specific tissue structure that allows for the smooth gliding of joints as well as allowing it to withstand the mechanical load of daily movements. Due to their avascular nature, damage to the articular cartilage is often associated with joint diseases that are notoriously irreversible with minimal repair, such as osteoarthritis (OA). As such, in order to improve on their therapeutical outcome, more is needed to understand the stem cell biology of the articular cartilage. Here we have discovered a new population of articular cartilage stem cell that is distinct to the embryonic articular progenitors. These stem cells have been shown to give rise to the articular cartilage in early postnatal development and importantly, can also be found in adulthood. These cells have displayed stem cells properties in vitro and are absent in old age indicating their potential role in maintaining the integrity of articular cartilage structure.

2.2 Statement of Authorship

Statement of Authorship

Title of Paper	Contribution of Gremlin 1 expressing stem cells to cellular architecture and stem cell biology of articular cartilage in development and adulthood.
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Jia Ng		
Contribution to the Paper	First author and main contributor. Concept design, literature search, review and formulation of primary draft, in addition to reviewing and incorporating co-author comments and suggestions.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	30/10/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Yan Ma		
Contribution to the Paper	Technical support.		
Signature		Date	25/10/2019

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Signature		Date	24/10/2019

Name of Co-Author	Danijela Menicanin		
Contribution to the Paper	Supervision and review of manuscript.		
Signature		Date	25/10/2019

Name of Co-Author	Daniel Worthley		
Contribution to the Paper	Investigation, conceptualisation, supervision and manuscript review.		
Signature		Date	30/10/2019

Name of Co-Author	David Haynes		
Contribution to the Paper	Supervision and manuscript review.		
Signature		Date	6/11/19

2.3 Introduction

Articular cartilage is hyaline cartilage located on the articular surface of bone. It is comprised of chondrocytes organised into a multi-zonal complex consisting of superficial, non-calcified and calcified cartilage surrounded by a rich extracellular matrix. Its primary function is to allow for smooth gliding of the articulating surfaces of the joint and to protect the subchondral bone form mechanical stress[1]. Collagen type II, aggrecan and additional macromolecules such as fibronectin make up the main components of the matrix and provide tensile strength and elasticity enabling the tissue to withstand compressive loads and shear forces from a range of motion in the joint[2]carter.

The developmental biology of articular cartilage derives from the prevailing model termed endochondral ossification. Cell condensation, presumably formed by mesenchymal stem cells, is initiated by a combination of tumour growth factor-beta (TGF-β), fibronectin and adhesion molecule N-cam. These condensations proliferate and differentiate into chondrocytes which then lay down the matrix that forms a scaffold for the osteoblasts to invade and form bone. As bone elongates, haematopoietic stem cells relocate from the liver to the bone marrow establishing a perisinusoidal niche with a supportive population of adult mesenchymal stem cells. Secondary ossification centres eventually form, creating the subchondral growth fronts at the end of the bone via cycles of chondrocyte hypertrophy, vascular invasion and osteoblast activity. Articular cartilage is finally established when the subchondral growth front approaches the articulating surface of the joint and stabilises, defining the thickness of the articular cartilage, and creating a compartment consisting of very specific stem-progenitor cells[3].

Historically, articular cartilage is known for its lack of regenerative ability upon challenges of injury, pathology or old age. This consequently leads to inevitable diseases such as osteoarthritis and rheumatoid arthritis. Ironically, despite the presence of a considerable number of stem and progenitor

cells in articular cartilage, tissue repair and regeneration continue to be minimal or non-existent. Significant effort has been placed into investigating ways to induce repair but to no avail, mainly attributed to the avascular nature of the tissue. In order for more successful reparative strategies to be developed, a better understanding of normal articular cartilage development is essential.

Early embryonic limbs are made up of continuous and uninterrupted chondrocytic cell condensation[4]. The first sign of articular formation is the appearance of compacted and flat mesenchymal cells at the prospective joint site named the interzone. As interzone appears where chondrocytes previously resided, it was proposed that they are the descendants of de-differentiated chondrocytes[5]. One of the earliest genetic markers of interzone cells is the growth and differentiation factor-5 (*Gdf5*)[4]. Recent advances using transgenic mouse models have shed light into the origin, fate and role of joint progenitor cells that may contribute to articular cartilage maintenance and endochondral growth[6,7]. By cell fate tracing, Gdf5-expressing cells marked a population of stem cells that gave rise to the articular cartilage, menisci, ligaments, epiphyses, synovium and fat pad[6]. This study is one of the few that highlight the existence of an embryonically defined progenitor cell population in the synovium that is distinct from those previously identified. Although Gdf5 marks developmental articular cartilage progenitor cells, it remains difficult to harness the therapeutic potential of these cells in adulthood.

The articular and growth plate cartilage are constituted by the same cell type (chondrocytes), however these structures are formed quite differently. Much is known about the formation and maturation of the growth plate cartilage, however, less is understood about the development of the articular cartilage[8-11]. Articular cartilage, unlike the growth plate which comprises of a cartilage template that is replaced by bone as the long bone elongates, establishes a permanent cartilage tissue. Although the development of articular cartilage is not fully understood, it is suggested that interstitial and appositional growth mechanism could explain the mechanism by which articular cartilage develops[7]. Both of these mechanisms require support of a progenitor cell population that would self-renew to repopulate the

superficial zone, as well as give rise to chondrocyte progenies that would make up the middle and deep zone of the articular cartilage. Studies to identify slow cycling stem cell populations, using metabolic labelling, have resulted in the discovery of two proliferative regions within the knee joint; one within the superficial zone of the articular cartilage, another within the subchondral plate[12]. It was shown that only the superficial progenitor cells in the articular cartilage gave rise to colonies with high colony-forming efficiencies[13]. Therefore, it would be logical to think that Lubricin, which is abundantly expressed by the chondrocytes in the superficial zone and encoded by the Prg4 gene, marks a population of articular cartilage progenitors in adulthood. Using the same lineage tracing technique in mice, two research groups highlighted the existence of articular cartilage progenitors limited to only the superficial zone and not the growth plate of young mice. The identified superficial articular progenitors further expanded into deeper regions of articular cartilage as the animals aged[7,14]. However, in later studies of further characterisation of Prg4 articular progenitor cells in adult articular cartilage development, Decker et al found that although Prg4 cells gave rise to the articular cartilage cells, Prg4 articular chondrocytes did not support the model of oppositional growth. On the contrary, articular cartilage growth and thickening relied on formation of non-daughter cell stacks and cells rearrangements with limited contribution by cell proliferation from the Prg4 lineage cells[2]. In light of these findings it is fair to hypothesise that a specific population of articular cartilage stem/progenitor cells, resident at different sites within cartilage, may be involved in sustaining the lateral expansion of postnatal articular cartilage.

Although chondrocytes localised to the growth plate hold a different role in skeletogenesis, they continue to provide a sustainable source of cells pivotal to bone elongation throughout adulthood. This population of cells may hold the capacity to regenerate articular cartilage upon challenge. Interestingly, it was recently shown that the resting zone in the growth plate houses a unique population of skeletal stem cell. These stem cells marked by the expression of parathyroid hormone-related protein (PTHrP) gave rise to skeletal tissues such as bone, cartilage and fat in vitro, and maintained the growth plate by interacting with the hypertrophic chondrocytes[15]. The PTHrP-expressing stem cells were not the first stem cell population, exhibiting chondrogenic differentiation potential, identified within the site of the

growth plate. Worthley et al identified a population of skeletal stem cells named the Osteochondroreticular (OCR) stem cells marked by the expression of Gremlin 1[16]. These stem cells are unique and distinguished from the traditional mesenchymal and the PTHrP-expressing stem cells as they have the potential to self-renew, form bone and cartilage but lack the ability to differentiate into adipose tissue. The distinguishing difference between OCR stem cells and the PTHrP-expressing stem cells is the demonstrated ability of OCR stem cells in giving rise to articular chondrocytes in vivo where this hasn't been confirmed for PTHrP-expressing stem cells. Could these OCR stem cells then be the key to a sustainable population of adult stem cells that play a pivotal role to articular cartilage repair?

2.4 Materials and Methods

2.4.1 Mice

The following mouse colonies were used in the study:

LepR-cre[17], *Acan-creER*^{T2}[18], *Grem1-creER*^{T}[16], *R26-LSL-TdTomato*[19] and *R26-LSL-ZsGreen*[19] are from Jackson Laboratory. All animal experiments were approved by the Animal Ethics Committee at the South Australia Health and Medical Research Institute (SAHMRI) under ethics number SAM189. All of the data sets described in the present study were gathered and verified across a minimum of 4 independent experiments.

2.4.2 Tamoxifen administration

Tamoxifen (#T5648, Sgima) for creER^{T} mouse lines was administered at Day 4 – 6 of age for developmental studies. Pups were injected subcutaneously with 2mg of tamoxifen dissolved in peanut oil. For adult and old age mouse inductions, 4 x 6mg doses of tamoxifen dissolved in peanut oil were administered on consecutive days within a week by oral gavage.

2.4.3 Articular Cartilage Lineage Tracing

For developmental tracing, 2mg of tamoxifen was administered by subcutaneous injection as a once off to P4 – P6 old mice. Following tamoxifen administration, the animals were sacrificed at incremental stages including 24 hr, 1 week, 1 month and 5 months. For adult tracing, 4 x 6mg of tamoxifen was administered by oral gavage to 6-week-old mice. The animals were humanely sacrificed at incremental time points post tamoxifen administration including 1 week, 1 month and 3 months. For tracing in old age mice, 4 x 6mg of tamoxifen was administered by oral gavage to 6 -7 month old mice and the animals were humanely sacrificed 1 week later.

2.4.4 Histology

Mice were humanely sacrificed before bones from both hind limbs were collected and all muscles removed before fixing in 4% paraformaldehyde overnight, decalcified in Osteosoft® (#101728, Millipore) for 3 – 4 days and dehydrated in 30% sucrose at 4°C before embedding in OCT compound (Sakura Tissue-Tek). Embedded tissues were stored at -80°C. 10μm frozen sections were collected on cryofilm (type IIC, Section-Lab) for staining. 0.04% toluidine blue (#198161, Sigma) in 0.1M sodium acetate pH4.0 and 0.1% fast green (#F7252, Sigma) in MilliQ water were used to demonstrate histological features of cartilage and bone.

2.4.5 Imaging

Stained sections were scanned using the 3DHistech Pannoramic 250 Flash II to generate brightfield images. Fluorescent images were captured either on the Olympus IX53 inverted microscope or the Leica TCS SP8X/MP confocal microscope.

2.4.6 Immunohistological and fluorescent staining

Immunohistochemistry and immunofluorescent staining were completed on 10μm frozen sections prepared as above. Antigen retrieval was performed by placing slides in a steamer submerged in antigen unmasking solution (#H-3300, VectorLab) for 6 min. Immunohistochemistry slides were treated for endogenous peroxidase activity by incubating in 3% H_2O_2 for 30 min. Blocking was performed in 2% BSA, 5% normal goat and 5% normal donkey serum. The following antibodies were used: anti-PCNA (#ab18197 Abcam, 1:200), ColX (#ab58632 Abcam 1:200), aSMA (#ab5694 Abcam, 1:400), OCN (#ab93876 Abcam, 1:200), Lubricin (#ab28484 Abcam, 1:100) and Sox9 (#AB5535 Millipore, 1:400). After overnight incubation at 4°C, slides were washed with PBST and incubated with species-appropriate secondary antibody (1:200) at room temperature for 1 h. Finally, slides were counter stained with DAPI before mounted with cover slip. For immunohistochemistry, after overnight incubation at 4°C, slides were washed with PBST and incubated with anti-rabbit biotin (#BA-1000, VectorLab 1:250)

at room temperature for 1 h and then streptavidin-HRP (#SA-5004, VectorLab 1:100) at room temperature for 30 min and developed with DAB chromogen (#K3468, Dako).

2.4.7 Cell culture

Both hind limb bones (femur and tibia) were collected from 7-week-old mice that had been subjected to tamoxifen 1 week earlier (ie tamoxifen administered at 6 weeks of age). The knee joints were dislocated at the epiphyses from both femur and tibia, gently disrupted using a mortar and pestle, and digested in 4mg/ml collagenase IV (#17104019, Gibco) and 3mg/ml Dispase (#17105041, Gibco) in α-MEM (#M5650, Sigma). Collected cells were then plated in complete media (α-MEM supplemented with 20% defined bovine serum, 100mM L-ascorbate-2-phosphate, 1mM sodium pyruvate, 50μg/ml streptomycin, 50U/ml penicillin, 2mM L-glutamine and 10μM Y-27632) and allowed to reach confluency before being sorted for lineage-traced cells (TdTomato positive). Cells were sorted based on the forward and side scatter plots, single cells as well as positive for TdTomato fluorescence. Live and dead (DAPI) staining was omitted as cells were later plated for expansion. Sorted cells were seeded at 1000 cells per 10cm dish to allow for isolation of individual clones using clonal cylinders (#Z370789, Sigma). Clones (single cell colonies) were then allowed to expand before performing clonogenicity and multilineage differentiation assays.

2.4.8 Assessment of clonogenicity and multilineage differentiation potential

For colony-forming unit – fibroblasts (CFU-F) and differentiation analysis, single recombined clones were isolated with cloning cylinders and then expanded and passaged for CFU-F and multilineage differentiation assays. CFU-F assay was performed by seeding cells at clonal density (1000 cells per 10cm dish) and subsequent culture in complete media for 14 days. The cultures were stained with 0.1% Toluidine blue in 2.4% formalin solution and the total number of colonies were counted where an individual colony was defined as ≥50 cells. The number of clones were reported as (CFU-F)/1,000 cells plated. Induction of osteogenic, chondrogenic and adipogenic differentiation was conducted by

maintenance of cell cultures in differentiation media (StemPro). Positive and negative assessment of differentiation potential was performed by staining cells with Alizarin red (osteogenesis), Alcian blue (chondrogenesis) and Oil Red O (adipogenesis). All in-vitro assays reported in this study were conducted on clonal cell populations of passage 5 or lower.

2.4.9 RNA isolation and RT-PCR

Total RNA was isolated from cells using TRIzol (Thermofisher) as per manufacturer's instruction. Complementary DNA (cDNA) was generated using SuperScript IV reverse transcriptase kit (Invitrogen) according to manufacturer's protocol. Transcript levels were assessed by QuantStudio 7 (Thermofisher) using IDT probes GAPDH (Mm.PT.39a.1) and Grem1 (Mm.PT.53a.31803129).

2.4.10 Statistical Analysis

All analyses were performed using Prism 8 (GraphPad software Inc.).

2.5 Results

2.5.1 Cellular architecture of articular cartilage is different in development compared to adulthood and old age.

Our findings indicate that at 1 week, the mouse joint is still undergoing endochondral ossification with no zonal organisation of the articular cartilage and bone formation in the developing limbs. The articular cartilage appears thin and highly cellular consisting of small and closely-bound cells and is rich in proteoglycan produced by the chondrocytes, demonstrated by deep purple staining of toluidine blue (Figure 1A). This is consistent with the prevailing model of skeletal development where chondrocytes are foundational to the process by laying down the matrix for the subsequent invasion of osteoblasts to form bone. Joint development matures in adulthood where chondrocytes organise themselves into multiple zones in the articular cartilage together with the subchondral bone and marrow space. Fast green (green) and toluidine blue (purple) staining of a 4-month-old mouse joint clearly outlines the separation of the subchondral bone and cartilage in the articular area and the growth plate. At this stage the articular cartilage is distinctly separated into the superficial zone, non-calcified and calcified zones.

It is widely believed that the lack of reparative potential in the articular cartilage is partly due to low proliferative capability of chondrocytes, resulting in an ability to renew themselves in adulthood and old age. Comparative genetic profiling has indicated that mature cartilage is genetically predisposed for functional transition in order to withstand biomechanical stresses whereas developing articular cartilage has genetic characteristics indicative of tissue growth and expansion[20]. Localisation of PCNA (proliferating cell nuclear antigen) showed a significant decrease in the number of proliferating cells in the articular cartilage with age in the following increments from development, (1 week) to adulthood (4 months) and old age (7 months) (Figure 1B). This is consistent with previously reported findings and may contribute to the limited repair and regenerative processes resulting from the low cellular turnover in adulthood and in old age. Not surprisingly, there was no significant difference in chondrocytes proliferation between adulthood and old age. This finding confirms that articular cartilage does not

undergo significant change once it has matured. Percentage of PCNA (brown) cells was calculated as a ratio of total number of cells in the articular cartilage (counter stained using haematoxylin stain). Articular cartilage zone in 1-week old animals was defined by the small and closely-bound cells near the articular surface.

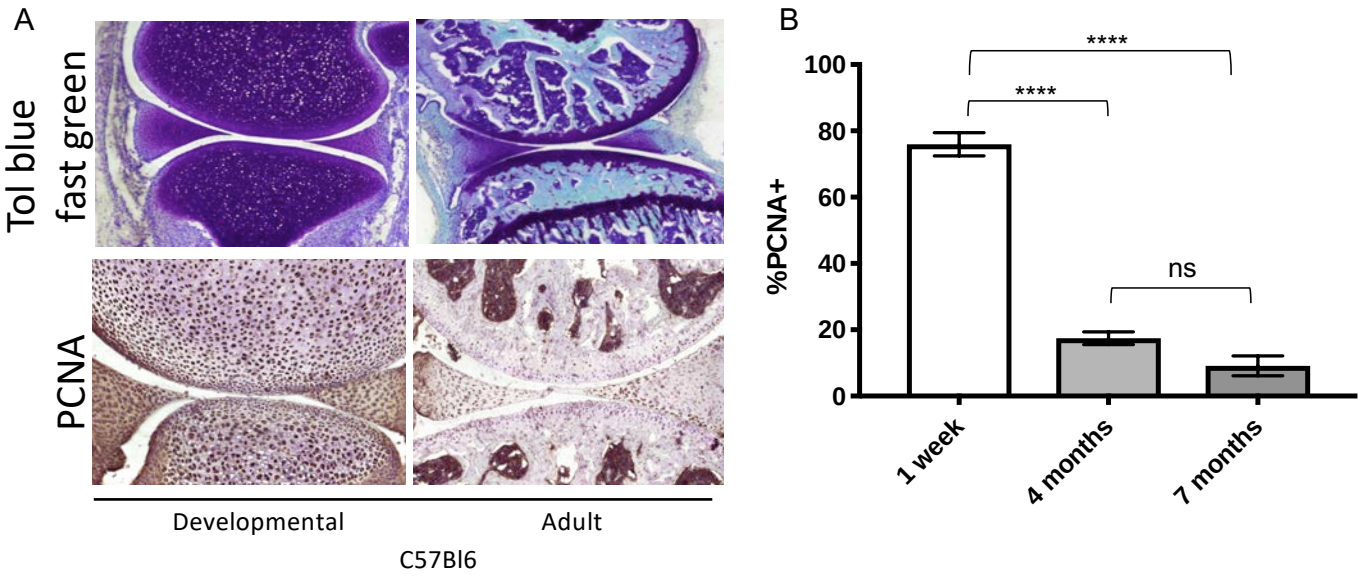


Figure 1. (A) Toluidine blue and Fast Green staining shows the articular cartilage and bone histology of postnatal developmental (1 week) and adult (4 months) bone. Toluidine blue staining indicates the proteoglycan production in the cartilage and Fast Green staining shows the bone in contrast to the cartilage. PCNA staining demonstrates the difference in chondrocyte proliferation between developing cartilage and mature cartilage. (B) Quantification of chondrocyte proliferation in articular cartilage at different stages. Percentage of PCNA+ cells was calculated as a total of the combination of all three zones (superficial, non-calcified and calcified) and a minimum of n=3 independent samples per age group were used to determine statistical significance.

2.5.2 Grem1 stem cells are articular cartilage stem cells during development.

Aggrecan (Acan) is the most well studied of proteoglycans as it is central to endochondral ossification processes and cartilage function in skeletal development[21]. It is believed that the traditional mesenchymal stem cells marked by the expression of LepR (Leptin receptor) give rise to cartilage because of their in vitro multilineage potential. We decided to investigate the role of the newly discovered Gremlin 1 (Grem1) expressing stem cells and LepR expressing stem cells in articular cartilage during development in comparison to Acan expressing populations.

Using a transgenic mouse model to mark different cell populations, distinguished by genetic expression of Grem1, LepR and Acan, we were able to trace them and their progenies to understand the interplay of stem cells within articular cartilage. Grem1 and Acan mice were administered a dose of tamoxifen at P4 – P6 of age and traced for 24 hours, 1 week, 1 month and 5 months. At 1st collection point of 24 hours post induction, Acan marked a large population of cells particularly in the tibia and meniscus in developing animals. On the other hand, at 24 hours, Grem1 marked a much smaller population of cells mainly restricted to the femur. LepR showed no tracing at P5 – P7 of age.

At the 1-week chase, both Grem1 and Acan cells proliferated and expanded, occupying chondrocytes in the articular surface as well as secondary ossification centers that eventually form bones. Not surprisingly, Acan marked all present chondrocytes within the articular cartilage structure and meniscus of the knee. Interestingly, although Grem1 marked the majority of articular surface chondrocytes, it only gave rise to a small population of cells in the meniscus.

The one-month chase demonstrated that Acan expressing cells gave rise to most of the structures in the joint including the multiple articular cartilage zones, osteocytes, perivascular cells and the meniscus. Although Grem1 cells gave rise to chondrocytes that expanded into multiple zones of the articular

cartilage, osteocytes, reticular cells and mature bone, the tracing pattern in the growth plate appeared clonal due the columnar arrangement of deposited cells. LepR-lineage cells continued to show no tracing of chondrocytes in the joint at about 1 month of age but identified as a precursor to multiple perivascular cells and the occasional cell in the synovium.

Consistently with previous data, by 5 months, Acan and Grem1 progenies stopped expanding and exhibited a significant decrease in cell proliferation within the knee as the mice aged. There was an expansion of Grem1 progeny cells within the calcified zone of the articular cartilage and an increase in mature bone cells. This finding highlights the potential contribution of Grem1 cells to vertical tissue growth, required for increase in long bone length and epiphyseal expansion and enlargement to provide adequate biomechanical function for the growing body[5].

Acan-expressing cell progenies displayed similar reduction in cell numbers in the calcified cartilage and an increase in mature bone cells. Tracing of LepR-expressing stem cells in 5-month-old mice indicated that LepR+ stem cells contributed to perivascular cells present in the subchondral marrow space as well as the synovial lining, but minimal contribution was apparent to the population of chondrocytes in the articular cartilage. Contrary to the traditional belief that mesenchymal stem cells give rise to chondrocytes, we have shown that LepR stem cells do not contribute to articular cartilage development.

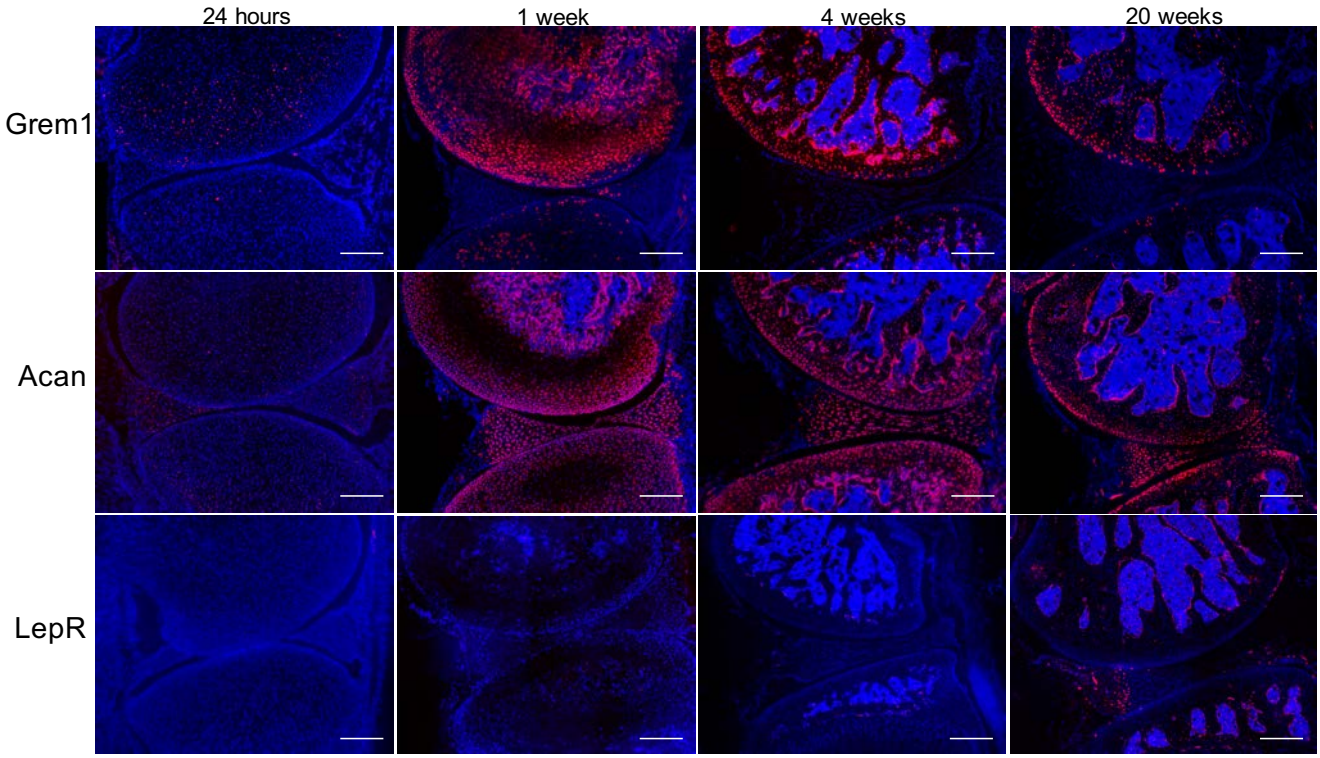


Figure 2. Lineage tracing of Grem1, Acan and LepR mice with one dose of tamoxifen administered at P4 – P6. Hind limbs were collected at 24 hours, 1 week, 1 month and 5 months post induction to identify cells marked by Grem1, Acan and LepR expression. Of note, LepR is a constitutive cre and therefore did not require tamoxifen induction. Scale bar = 200μm

2.5.3 Postnatal Grem1 stem cell contribution to articular cartilage.

At 5 months, articular cartilage in mice is separated into 3 zones, namely; the superficial zone composed of elongated, flattened cells oriented parallel to the articular surface. The non-calcified zone, adjacent to the superficial zone consisting of larger and rounder chondrocytes oriented more randomly and often aligned in vertical stacks perpendicularly to the articular surface. Lastly, the deepest calcified zone, where cartilage matrix is calcified, separated from the other zone by the tideline where cells are scarce, and chondrocytes are hypertrophic[22] (Figure 3A). Percentage of Grem1, Acan and LepR cells contributing to the different zones of articular cartilage were quantified as the ratio of TdTomato positive cells (red) to DAPI (blue) within each of the respective zones (Figure 3B). Quantification assays revealed that Acan expressing cells contributed significantly to all three zones in the articular cartilage compared to Grem1+ and LepR+ cells. In comparison, Grem1 expressing cells contributed to a significantly higher number of cells in the non-calcified and calcified layer of the articular cartilage compared to LepR. This is reflective of the small number of chondrocytes in the superficial zone seen in 5-month-old LepR-traced animals. The finding further proposes that LepR cells may contribute to articular cartilage later in adulthood or the results are potentially concurrent with initiation of calcification and degeneration of the articular cartilage with age. Nevertheless, the number of LepR-expressing chondrocytes did not propagate to the other zones as seen in Grem1- and Acan-lineage traced animals. This further confirmed that LepR expressing mesenchymal stem cells do not contribute to the development of articular cartilage, thus explaining the poor efficacy of current mesenchymal stem cell based therapeutics in the long run as they fail to elicit regeneration of native articular cartilage with its distinct nature, architecture and multifaceted function[5].

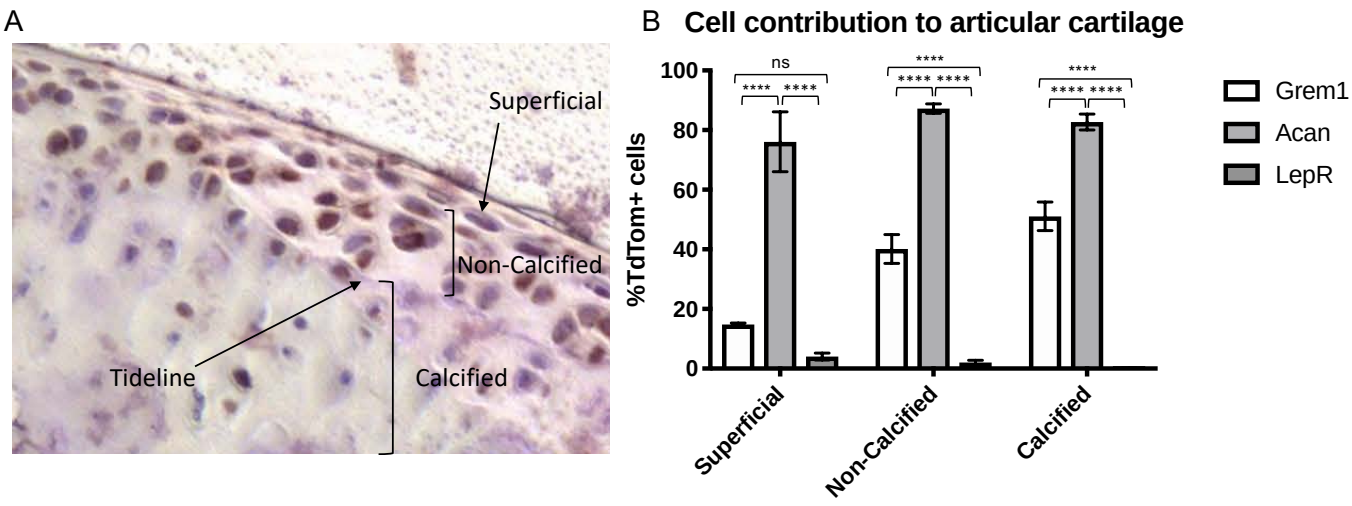


Figure 3. (A) Haematoxylin and Eosin staining of a 5-month-old knee showing separation of the different zones in the articular cartilage. (B) Quantification of the percentage of lineage traced cells marked by the expression of Grem1, Acan and LepR in 5-month-old mice. Cell distribution is quantified within the different zones highlighted in (A). Statistical comparison was done within the different zones, a minimum of n=4 independent samples per expression group were used for the statistical analysis.

2.5.4 Postnatal Grem1 stem cells gave rise to multiple different cell types in the joint.

Following on from lineage tracing of the Grem1, Acan and LepR cells, immunostaining was done to determine the different cell types that Grem1, Acan and LepR gave rise to during development. Immunostaining of the 5-month chased knee joint showed that Grem1 stem cells gave rise to articular cartilage cells marked by Sox9, hypertrophic chondrocytes marked by Collagen X (ColX) and osteoblast cells marked by osteocalcin (OCN). Grem1 also gave rise to a small population of fibrocartilage cells in the deeper zones and perivascular cells marked by alpha-smooth muscle actin (αSMA). Acan gave rise to all of the different chondrocytes within the joint with the exclusion of the synovial lining. On the other hand, LepR did not give rise to any population of chondrocytes marked by ColX and only a rare few of $Sox9^{+}$ chondrocytes. However, LepR expressing cells gave rise to a few fibroblastic-like cells (αSMA^{+}) in the synovium and osteoblasts (OCN^{+}) in the subchondral bone area. This data demonstrates that LepR stem cells do not give rise to cartilage but contribute to the formation of synovium and bone in the mouse articular joint. This further contributes to the rationale underlying the limited success of current stem cell therapy utilising traditional mesenchymal stem cells to sustainably repair and regenerate mature articular cartilage.

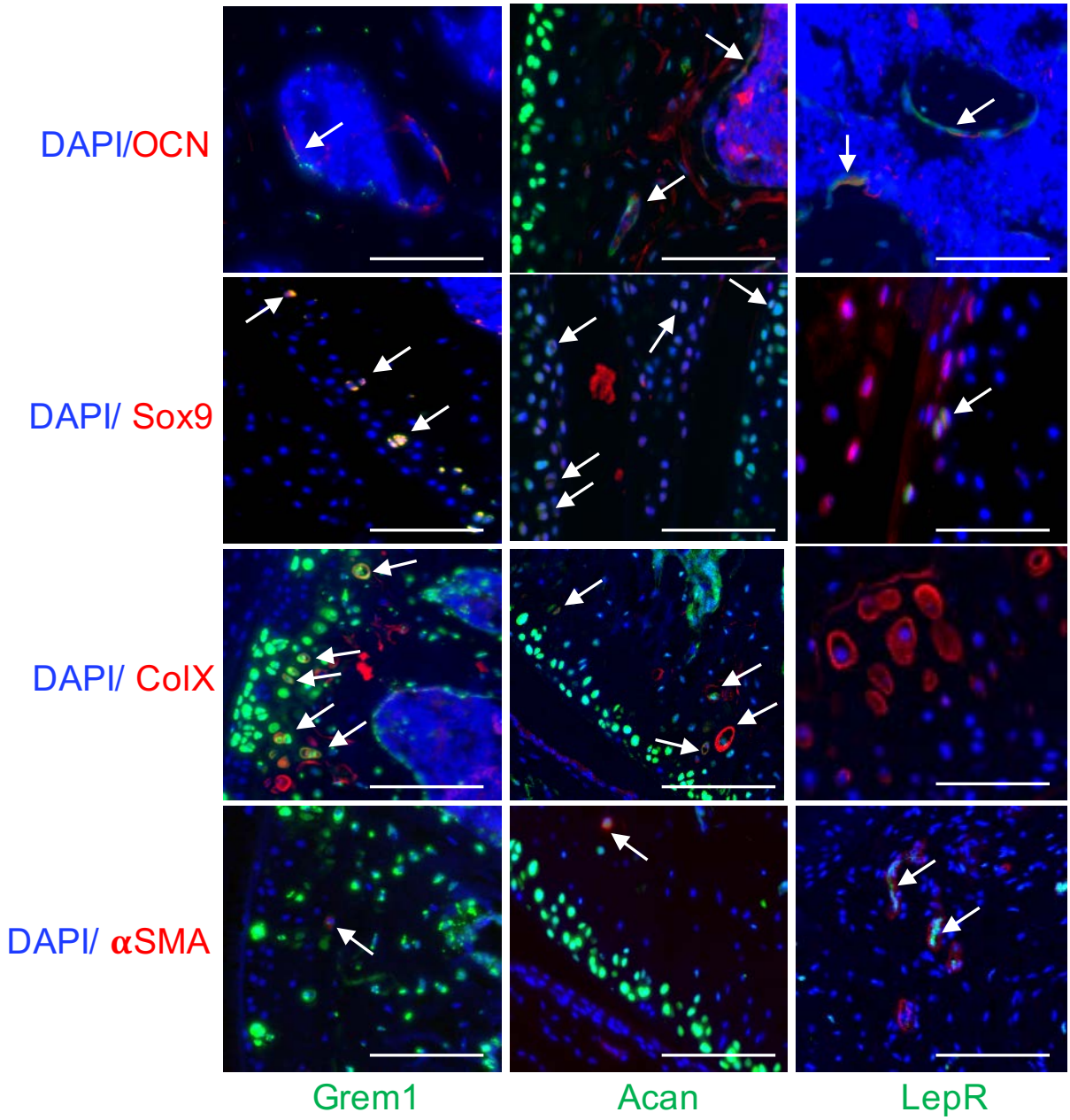


Figure 4: Characterisation of cell types from 20 week lineage traced animals of different population of target cells using immunofluorescence staining. OCN showing osteoblastic cells (top roll), Sox9 showing chondrogenic cells (second roll), ColX showing hypertrophic chondrocytes (third roll) and αSMA+ showing fibroblastic cells (bottom roll). Arrows indicate positive for colocalised staining. Analysis was done on a minimum of n = 3 independent samples. Scale bar = 100μm

2.5.5 Grem1 stem cells continue to exist in articular cartilage in adulthood.

Unlike previously, where tracing of Gdf5- and Prg4- expressing articular cartilage stem/progenitor cells was induced at embryonic stage, in this study Grem1-expressing articular chondrocytes were traced through adulthood. Grem1 mice were induced at 6 weeks of age and hind limbs were collected 1 week later, showing that the traced cells were mostly restricted to the superficial zone of the articular cartilage and the growth plate along with some reticular cells surrounding the subchondral bone. The 1-month chase experiments demonstrated continued tracing of cells in the superficial zone which extends into the middle zone of articular cartilage in the knees as well as a small number of chondrocytes in the growth plate (Figure 5). Consistent with previous reports, articular chondrocytes exhibited a slow rate of proliferation identified by the slow expansion of cells traced towards the deeper zones of the articular cartilage. This indicates that Grem1 may mark a population of cells with stem/progenitor potential and a role in tissue morphogenesis in adulthood however this hypothesis requires further characterisation. Unique to previously described articular cartilage progenitor cells, Grem1-expressing cells were detected in young adulthood where articular cartilage structure has matured, and the nature of the collagen matrix is permanent. With recent studies confirming the presence of progenitor-like cells in the superficial zone of adult articular cartilage, Grem1 may present as the marker for such populations. Further investigations are required to test the stem cell potential of these cells in adult mouse knees.

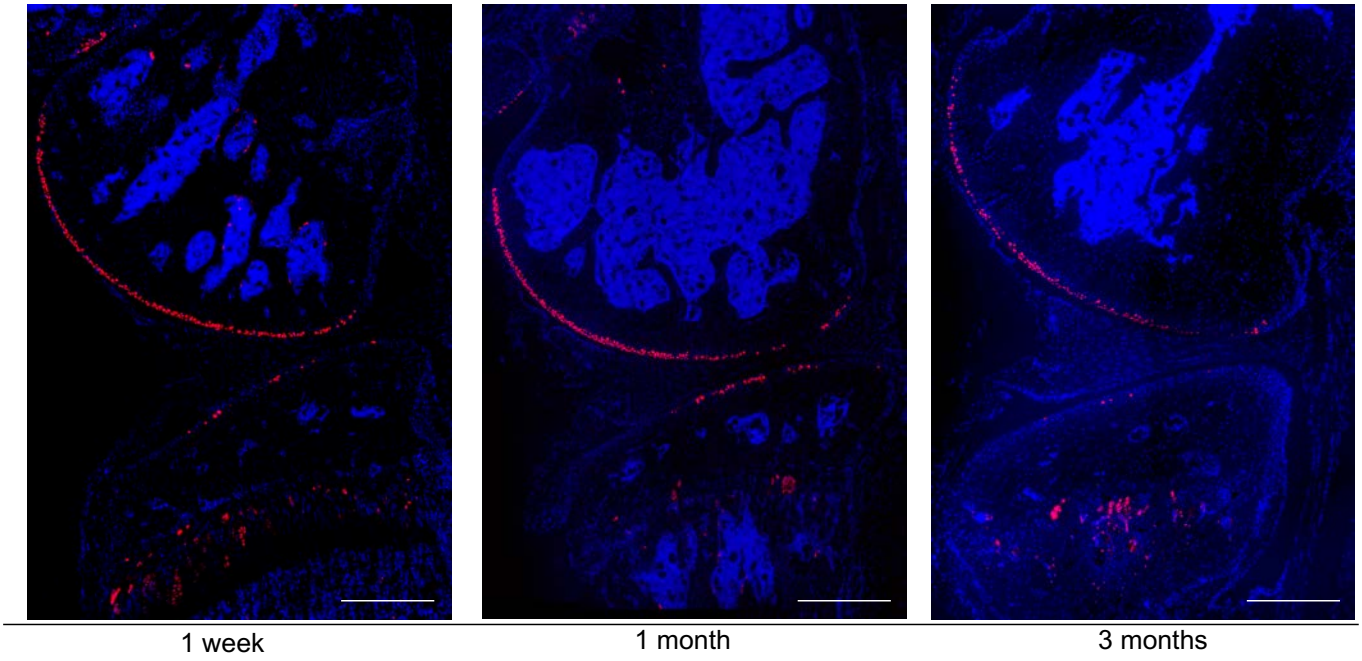
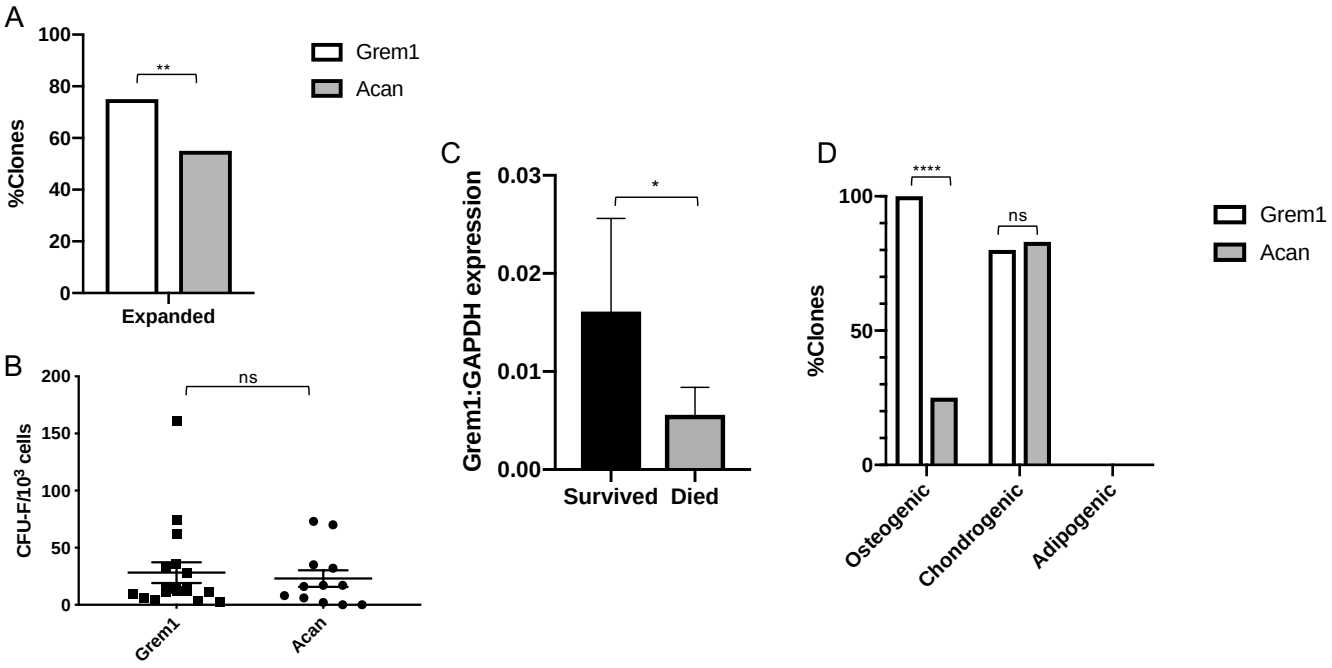


Figure 5. Lineage tracing of young adult Grem1 knees where 4 doses of tamoxifen were administered to 6-week-old mice. Knees were collected 1 week, 1 month and 3 months post tamoxifen induction for analysis. Representative figures of each collection time point with a minimum of n=4 independent samples were analysed per time point. Scale bar = 500μm.

2.5.6 A heterogenous population of adult Grem1 and Acan cells with different stem cell potential exist in the mature articular cartilage.

The clonal patterning of Grem1 and Acan expressing articular cartilage progenies suggests that not all Grem1 and Acan cells are the same. Articular chondrocytes may present a heterogeneous population of cells with subsets of cells committed to differentiation into mature lineages. To prove the theory that not all Acan or Grem1 traced cells are stem cells, we isolated these cells from the articular joints in the knees and subjected clones to CFU-F and multilineage differentiation assessment. As LepR-expressing cells did not give rise to any cartilage cells in young adult articular joints, LepR was excluded from these experiments. Cells digested from the epiphyseal joints were expanded in vitro before they were sorted for the expression of TdTomato. After 1 week of culture monoclonal populations were isolated using cloning cylinders and passaged into a 6-well plate. Clones were selected based on size (2.5mm – 3mm diameter or ~180 - 200 cells) after being in culture for no more than 2 weeks. The number of proliferating clones (defined by an expansion of >30,000 cells or >80% confluent in a 6 well plate) isolated from Grem1-lineage was significantly higher (>70%) in comparison with clones isolated from the Acan-lineage in the articular joint (>50%). The clones that survived did not show significant difference in clonogenicity potential between Grem1- and Acan-lineage cells as reflected in the CFU-F assay. qPCR was used to determine Grem1 expression between the proliferating and the non-proliferating clones of the Acan-lineage. The Acan-lineage clones that survived (>30,000 total cell number after 2 weeks expansion) expressed significantly higher levels of Grem1 in comparison to non-proliferating clones. This data highlights the role of Grem1 in self-renewal of the Acan-lineage clones. Clonal populations generated from Grem1- and Acan-lineage cells were induced to differentiate into osteogenic, chondrogenic and adipogenic lineages. Consistent with previously published data, none of the Grem1 nor Acan populations exhibited the capacity to differentiate into adipocytes. On the contrary, all of the Grem1 clones differentiated into osteogenic cells, whilst only 20% of the Acan populations displayed this capacity. There were no significant chondrogenic capability difference between the Grem1- (80%) and Acan- lineage (83%) clones. The results demonstrated that the articular joint houses a heterogenous population of cells with different clonogenicity and lineage differentiation potential.

Grem1 expressing articular cells also present as a mixed population of stem/progenitor cells with different stemness and multi-lineage capacity as demonstrated in our in vitro assays. Compared to Acan-lineage derived articular cells, Grem1 marked a unique subset of skeletal stem cells residing in the articular cartilage.



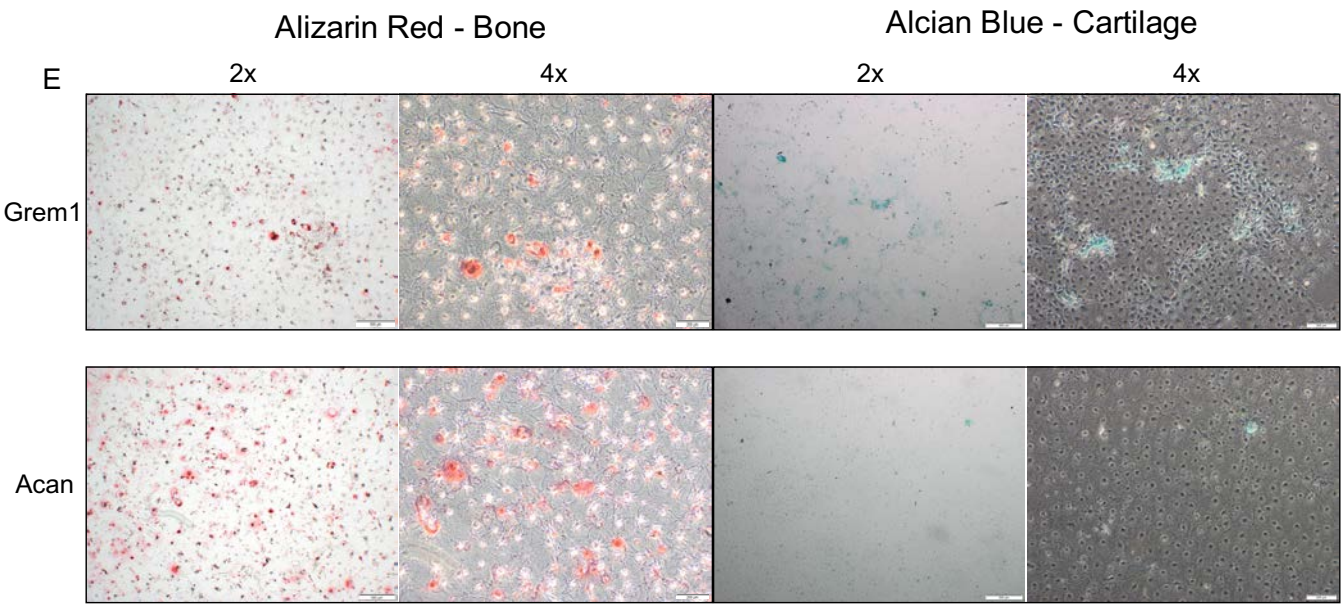


Figure 6. Sorted Grem1- and Acan-traced clones isolated using cloning cylinders were expanded for CFU-F and mutilineage differentiation assays. (A) Percentage of proliferating clones isolated that expanded indicating self-renewal capability. (B) The number of clones that expanded from a single isolated clone were put through CFU-F assay to determine clonogenicity. Colonies were quantified as total number of colonies (defined as ≥50 cells)/ 1000 cells plated in a 10cm dish. (C) qPCR of Grem1 expression as a ratio of GAPDH, of viable and proliferating clones versus non-viable clones. Significantly higher expression of Grem1 in the viable clones indicates that Grem1 expression may be necessary for the self-renewal capability of the Acan cells. (D) Clones that expanded and underwent multilineage differentiation showed that not all clones harbour the potential to give rise to different lineage cells. Of note, none of the clones from both Grem1- and Acan-traced cells differentiated into adipocytes. (E) A representative of Alizarin Red and Alcian Blue staining of clones indicating positive for osteocytes and chondrocytes differentiation respectively. A minimum of n=4 mice of both genders per cell population were used in this experiment. All cells used in this experiment were under passage 5 to minimise the effect of in vitro culture on cell phenotype.

2.5.7 Cell morphology indicates self-renewal capabilities.

Tracing of clones to determine self-renewal capabilities in vivo is technically challenging. In order to find identifiable features of clones with greater self-renewal capabilities, we decided to track the clones through in vitro expansion and CFU-F assays. Sorted Grem1- and Acan-lineage cells were photographed immediately before clones were isolated with clonal cylinders. In vitro, different clones varied in appearance and cell size. Clones containing fibroblastic-like cells resulted in higher CFU-F counts compared to those that appeared less fibroblastic in morphology. This was specifically evident in the Acan-traced clones in comparison to the Grem1-traced clones, indicating that the traditional fibroblastic-like morphology observed in MSCs could be used as a predictor of clonogenicity of the Acan and Grem1 cells. Having said that however, the same fibroblastic-like cell morphology cannot be applied as a determinant of the differentiation potential of the clones.

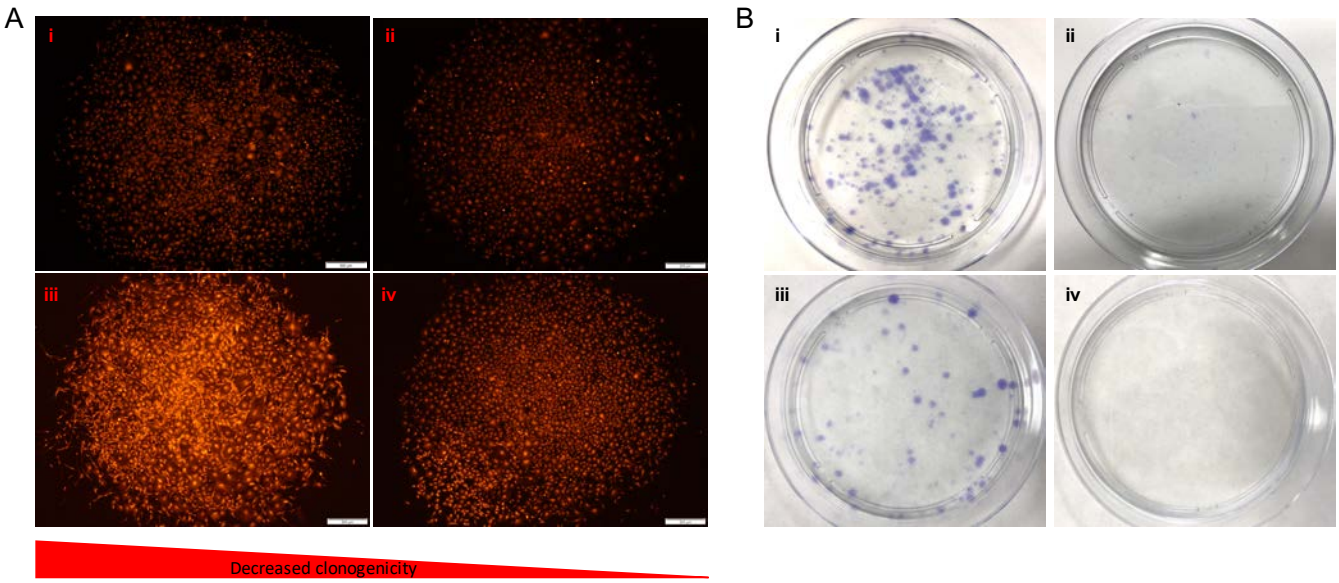


Figure 7. (A) Sorted Grem1- and Acan-Tdtomato traced cells clones were captured and monitored throughout clonogenic and multi-lineage induction assays. Clones that exhibited fibroblastic-like morphology appeared to have higher clonogenic capacity. (B) The same respective clones that underwent CFU-F assay.

2.5.8 Grem1 stem cell is absent in articular cartilage in old age knees.

Thus far, we were able to demonstrate that Grem1-expressing stem cells play a role in early postnatal development and maturation as well as articular cartilage morphogenesis in young adulthood, by lineage tracing. Conversely, Grem1-expressing mice where tamoxifen was administered at 7 months of age, to induce tracing of the Grem1-expressing articular cartilage cells, showed that Grem1-expressing stem cells rarely exist in the articular cartilage and appeared to significantly diminish with age. In addition to the articular cartilage, this loss of marked cells was also observed in the growth plate. Quantification of Grem1-expressing cells at 2 weeks (early postnatal), 2 months (young adult) and 7 months (old age) old showed a significant decreased in the number of cells in the articular cartilage only in the 7-months-old mouse knees. Toluidine blue staining of proteoglycan in the articular cartilage of aged mice indicated a loss in proteoglycan production and integrity, consistent with the natural wear and tear pathology in the elderly. We therefore make the assumption that the loss of Grem1-expressing stem cells within the articular cartilage may lead to a decrease in proteoglycan production and thus impact on the integrity of articular cartilage. Considering osteoarthritis is primarily a disease of old age, we assume that the loss of Grem1 stem cells may further have an important role in the disease onset and progression.

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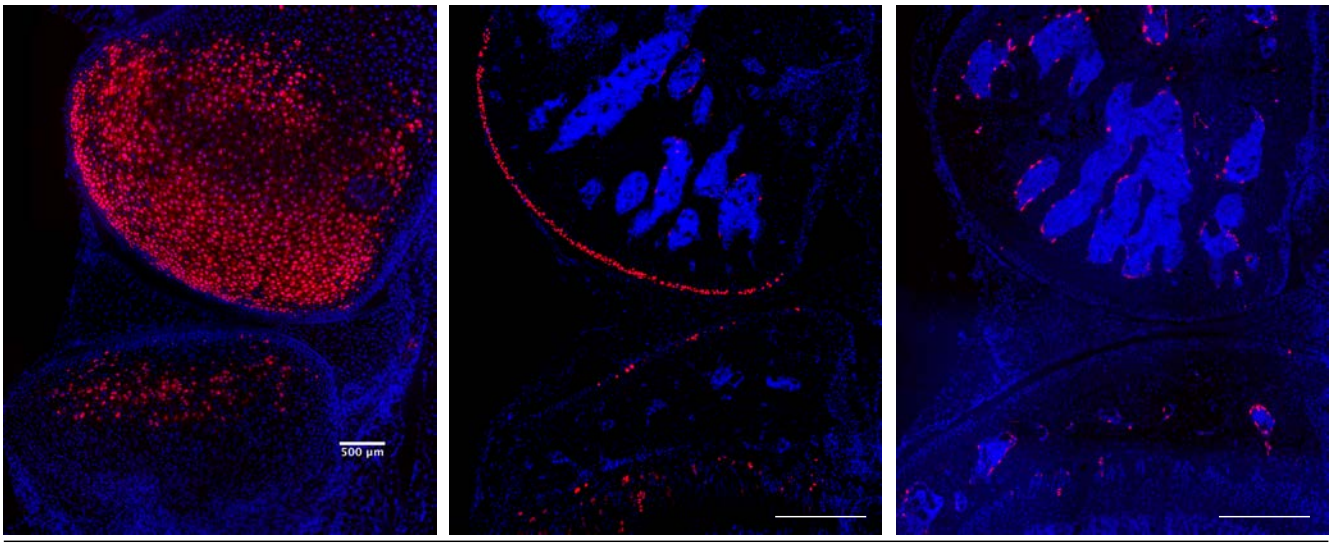

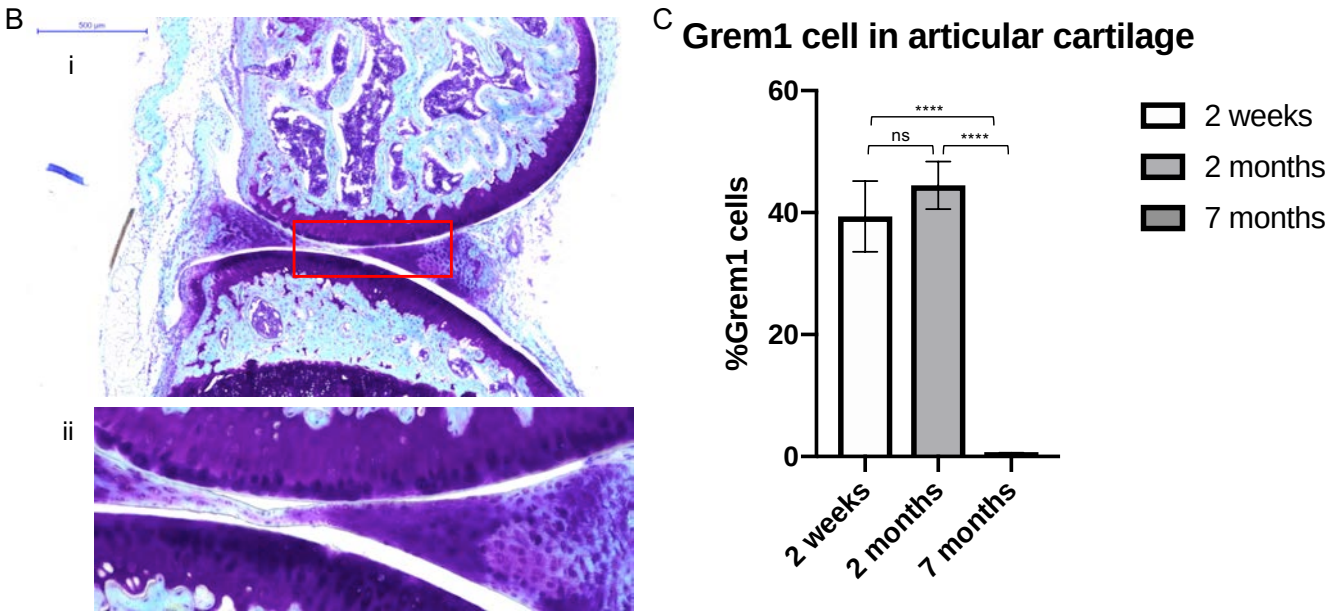

Figure 8. Tracing of Grem1-expressing stem cells through different stages of skeletal development. (A) Representative picture of Grem1 mice with 4 doses of tamoxifen administered at P6, 6 weeks and 7 months of age, collected 1 week post induction, respectively. (B) Representative staining of toluidine blue and fast green showing the diminished proteoglycan production in the articular cartilage of 7 month old mouse knee tissue (i) scanned at 4.9x magnification (ii) scanned at 14.5x magnification. (C) The quantification of Grem1 cell contribution to the articular cartilage through different stages of life. The number of Grem1 positive cells was quantified as a ratio of total number of cells in the superficial and non-calcified zones of the articular cartilage counter stained with DAPI (green boxed area in part A). A minimum of n=4 independent samples per age group were used for the statistical analysis in this experiment. Scale bar = 500μm.

2.6 Discussion

This study endeavoured to assess the developmental processes involved in the assembly of articular cartilage architecture postnatally and to shed light into the underlying stem cell biology of articular cartilage after birth. The function of the articular cartilage is fundamental to joint functionality and body movement. Our data highlighted the tremendous structural modification occurring from 1 week to 1 month of age that is required for cartilage to thicken and acquire distinct zonal organisation to better accommodate the functional requirement of adult animals. The limited proliferative ability of mature chondrocytes, together with the avascular characteristics of the articular cartilage, restricts tissue regeneration or repair.

A lot is known about the organisation, extracellular matrix and phenotypic properties of cartilage but cellular architecture and stem cell biology processes still remain to be elucidated. In these studies, we identified a population of stem cells marked by the genetic expression of Gremlin 1 (Grem1) that play a major role in postnatal articular cartilage maturation. Grem1, as a member of the bone morphogenetic protein (BMP) antagonist family, is highly involved in skeletal remodification[23-26]. BMPs are considered a group of pivotal morphogenetic factors required to orchestrate tissue architecture throughout the body, particularly in the formation of bone and cartilage[27]. Therefore, the demonstrated involvement of Grem1 expressing cells in articular cartilage morphogenesis is not surprising. Lineage tracing in this study, done during the early postnatal development of articular cartilage has shown that Grem1 expressing cells did not solely give rise to chondrocytes in the articular cartilage (marked by the expression of Sox9 and ColX) and growth plate, but also osteocytes (marked by OCN) and some perivascular cells (marked by aSMA) within the subchondral bone. Long-term chase experiments, conducted at 5 months-post induction indicated that the contribution of Grem1 cells within the different zones of the articular cartilage increased, in support of the assumption that Grem1 lineage cells are responsible for the appositional growth and thickening of the articular cartilage with age. Grem1 stem/progenitor cells contributed to 14%, 40% and >50% of the superficial, non-calcified and calcified

zones, respectively. This suggests that Grem1 may represent a population of superficial progenitor/stem cells, that in turn would give rise to other chondrocytes supporting the thickening of the articular cartilage with age. In comparison, the traditional MSCs marked by the genetic expression of LepR however, exhibited a limited role in early postnatal articular cartilage remodelling. Traditionally, MSCs were believed to be the stem cells that give rise to all skeletal tissues including articular cartilage because of their in-vitro stemness properties. In this study, by utilising a constitutive cre recombinase system driven by the expression of LepR, we have shown that MSCs do not give rise to cartilage tissue in vivo. LepR stem cells gave rise to perivascular cells, bone and synovial lining cells but chondrocytes within the growth plate as well as the articular cartilage were rarely found. Although the 5-month chase data showed some tracing of the LepR cells in the articular cartilage indicative of a possible role in mature adult/old age articular cartilage maintenance, these cells remain limited, contributing to only <5% of the superficial, 2% of the non-calcified and 0% in the calcified zone. Aggrecan (Acan) was used in the experiment as a baseline comparison of the contribution of cartilage cells to skeletal development. Acan is a major structural macromolecule of cartilage that serves as a primordium of most bones during development, with the mutation of Acan being perinatally lethal[28,29]. It has previously been reported that mice with Acan mutations have cartilage largely constituted by chondrocytes and lacking in matrix which results in abnormal cellular architecture of cartilage and endochondral ossification[30]. In 2-week old transgenic mice, induced at the same time points, the number of Grem1 expressing cells in endochondral ossification is comparable to that of Acan expressing cells, indicative of the importance of Grem1 during development.

Recent studies have confirmed the presence of cells with progenitor or stem characteristics in the superficial zone of adult articular cartilage[13,31,32]. Our investigation further identified that the Grem1 expressing cells are not only important for endochondral ossification process that give rise to the articular cartilage in development, but also contribute to the morphogenesis of adult articular cartilage. Lineage tracing in young adulthood showed that Grem1 marked a population of chondrocytes that are confined in the superficial layer of articular cartilage in mice. These superficial cells again gave rise to

chondrocytes in the deeper zone of the articular cartilage with time, implying the role of Grem1 expressing cells as a stem/progenitor population in adult articular cartilage. Our in vitro study verifies the stem cell properties by showing the self-renewal and differentiation capabilities of the Grem1 expressing cells isolated from the mouse epiphyseal joints within the knees in adulthood. Although the CFU-F assays did not demonstrate a significant difference between the Acan- and Grem1-expressing clones, qPCR data comparing the expanding Acan clones to those that lacked proliferative capacity highlighted that Grem1 expression may be necessary for the self-renewal properties of the Acan clones. Evidence of the limited differentiation potential of the Acan clones also suggests that Acan marked a population of progenitor cells that are highly committed to differentiating down the chondrocytic lineage in comparison with Grem1 expressing clones which mainly marked stem cells of self-renewal and multilineage capabilities.

Limited intrinsic repair capacity of mature articular cartilage is widely appreciated, and major efforts have been directed to understand the changes within the articular cartilage. Here, we show that Grem1-expressing cells are major contributors to articular cartilage at both developmental and adult stages. The adult articular cells gave rise to cells in the deeper layers of chondrocytes in the articular cartilage but also further display progenitor/stem cell characteristics in vitro. Interestingly, in articular cartilage of aged mice Grem1-expressing cells can no longer be found with lineage tracing, showing <1% of them remaining in all three zones of the tissue. With osteoarthritis (OA) primarily considered an old age disease, it can be assumed that the Grem1-expressing progenitor/stem cells may contribute to the progression of OA. Further studies are required to investigate the role of Grem1-expressing progenitor/stem cells in disease of cartilage tissue.

Our investigations demonstrate existence of a population of stem/progenitor cells that not only give rise to articular cartilage in development but also in young adulthood. This is the first study that has identified a possible marker in isolating these progenitor/stem cells. With limited success in current cell

therapy for OA, our study has shed some light into the possible explanation of the modest sustainable repair by the traditional MSCs. The identification of these Grem1-expressing cells has enhanced our understanding of the stem cell biology of articular cartilage. Inclusion of Grem1-expressing progenitor/stem cell population in bioengineering constructs and cell therapy could offer novel therapies that may be the key to successful cartilage regeneration and repair in cartilage disease and injury.

2.7 Conclusion

Grem1 marks a novel population of articular cartilage chondrocytes presenting with progenitor/stem cell characteristics. Further investigation and stringent characterisation of this cell population in cartilage disease may offer insight into their potential therapeutic capacity and cell based regenerative and repair application for cartilage tissue.

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Chapter 3: The role of Gremlin 1 expressing stem cells in Osteoarthritis.

Jia Q. Ng, Christopher Little, Mari Suzuki, Laura Vrbanac, Yan Ma, David Haynes, Danijela Menicanin, Daniel Worthley

3.1 Abstract

OA is a disease of the whole joint that has a prominent impact on patients' quality of life. Being the most common form of arthritis, the economic burden of this disease is estimated to increase with the rise in the current aging and obese population worldwide. Despite its significant impact, treatment remains limited even with the promise of stem cell-based therapy. The molecular aspect of OA progression is well understood, however, little is known of the articular cartilage stem cell biology and their involvement in OA. This study investigates the role of different skeletal stem cells in the progression of OA, in particular the newly discovered Grem1-expressing articular stem cells and their role in OA development. Using transgenic mice to trace the fate of the different population of skeletal stem cells, we uncovered that Grem1-expressing articular stem cells are lost in OA progression. Single-cell RNA sequencing found that these cells isolated within the articular cartilage possessed increased expression of Prg4, a previously reported articular progenitor marker. These indicated that Grem1-expressing articular stem cell may be a potential therapeutic target to delay or even reverse the progression of OA.

3.2 Statement of Authorship

Statement of Authorship

Title of Paper	Contribution of Gremlin 1 expressing stem cells to cellular architecture and stem cell biology of articular cartilage in development and adulthood.
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Jia Ng		
Contribution to the Paper	First author and main contributor. Concept design, literature search, review and formulation of primary draft, in addition to reviewing and incorporating co-author comments and suggestions.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	30/10/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Christopher Little		
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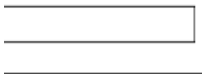
Signature		Date	25/10/2019

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Contribution to the Paper	Supervision and review of manuscript.		
Signature		Date	25/10/2019

Name of Co-Author	Daniel Worthley		
Contribution to the Paper	Investigation, conceptualisation, supervision and manuscript review.		
Signature		Date	30/10/2019

Name of Co-Author	David Haynes		
Contribution to the Paper	Supervision and manuscript review.		
Signature		Date	6/11/2019

3.3 Introduction

Osteoarthritis (OA) is a disease of the synovial joint characterised by clinical symptoms and distortion of periarticular tissues including muscles, ligaments and bone[1]. It is a costly, common and debilitating condition arising from genetic, metabolic, biochemical, inflammatory and mechanical factors[2]. Central to the pathogenesis and diagnosis, is the degeneration of the articular cartilage in the joint[3]. OA is the most common form of arthritis with prevalence estimated at 220 million people worldwide[4]. It is classified into two groups; 1) localised or generalised primary OA, more commonly found in post-menopausal women, and 2) secondary OA resulting from trauma, obesity, Paget's disease, or inflammatory arthritis[5]. Cost of the disease burden of OA is estimated to account for between 1% and 2.5% of the gross national product in USA, Canada, UK, France and Australia[4]. With an increase in aging population and obesity, personal and economic burden is expected to grow significantly in the coming decades.

OA is most commonly associated with aging, however, secondary causes such as injury and contributing risk factors including obesity or genetic predisposition exacerbate the severity and progression of the disease[5]. The first pathologically identifiable feature of OA is the progressive degeneration of articular cartilage. This degeneration then leads to inflammation, subchondral bone destruction, osteophyte formation, loss of muscular and ligamentous support and eventually the defining symptoms of joint pain, instability and stiffness[6].

Articular cartilage is localised to the articular surface of the bone, constituted by chondrocytes and surrounded by an intrinsic network of extracellular matrix with dense collagen fibre and proteoglycan contents. It consists of the superficial, middle and calcified zones populated by chondrocytes that are organised in different orientations to provide resistance to compressive forces. Its main function is to allow for smooth gliding of the articulating surfaces of the joint and to protect the subchondral bone

from mechanical stress[7,8]. The integrity of the articular cartilage depends solely on resident chondrocytes embedded within the extracellular matrix (ECM) responsible for maintenance of tissue homeostasis[9] along with the support of surrounding growth factors, cytokine enzymes and transcription factors[10-13]. Compromised chondrocyte function leads to matrix degeneration and cell senescence with apoptosis, therefore, the associated changes in communication between the immune system, cartilage and bone drive advancement of the disease. OA is pathologically characterised by flaking and fibrillation of the articular cartilage surface and destruction of the cartilage microarchitecture[14]. Early studies suggested that cartilage repair may occur through the proliferation of endogenous cartilage cells[15,16], but this proliferative repair, nonetheless, becomes more challenging in the elderly where articular chondrocyte proliferation has been shown to significantly decrease with age[17].

Considering the significant impact of OA on the world's population, the current lack of treatment strategy to reverse or delay disease progression is concerning. Current therapies focus on pain relief and long-term management of symptoms, which constitute modification of lifestyle factors (weight loss, exercise and dietary supplements) integrated with pharmaceutical analgesia or anti-inflammatory agents and ultimately escalating to joint replacement surgery, as the last resource[18]. Joint replacement surgery is invasive and costly, and often associated with significant morbidity[19]. Substantial efforts have been placed into exploring potential induction of endogenous cartilage repair through microfracture and osteochondral grafts[20,21]. While these applications provided some encouraging results in providing temporary relief of pain, these treatments cannot be applied to more extensive damage in the joint[20-22]. As such, focus has shifted toward drugs that promote chondrogenesis and osteogenesis, including matrix degradation inhibitors, apoptosis inhibitors, and anti-inflammatory cytokines[23]. These drugs however, showed varying levels of efficacy and lacked reproducible success in preventing disease development and progression[24].

Pathological processes presented in OA highlight the disease as a promising candidate for utilisation of regenerative cell therapy, as the loss of articular cartilage is central to disease development. Further to this, the articular cartilage is located within an immuno-tolerant tissue compartment, accessible for cell delivery. Cell-based therapies, using autologous chondrocytes, have been tested since the 90s[15]. In brief, such protocols have involved extraction and expansion of chondrocytes ex vivo, followed by autologous transplantation into the defective joint[15]. The expanded chondrocytes, however, lose their chondrocytic phenotype and proliferative capacity thus prompting the need for a more accessible source of cells with a greater expansion potential[25]. Mesenchymal stem cells (MSCs) possess the potential to differentiate towards bone, cartilage and adipose tissue and have long-term self-renewal capabilities[26]. More interestingly, MSCs exhibit further therapeutic efficacy via immunomodulatory mechanisms due to the lack of human leucocyte antigen (HLA) class II expression. The immunomodulatory properties in combination with their paracrine activity and secretion of bioactive molecules, hold a pivotal role in regenerative capacity held by these cell populations[27]. The most common source of MSCs for OA therapy are bone marrow, adipose and synovial tissues. Conclusive and reproducible results from preclinical studies have been confronted with numerous challenges due to vast deviations between animal disease models of OA[28-31]. The inconclusive results from these studies can further be attributed to the cellular heterogeneity of in vitro expanded MSCs isolated from multiple tissue sources[32]. It is widely presumed that MSCs are the developmental origin of all skeletal tissues[33]. Several groups have since suggested that despite their in vitro lineage repertoire, they may not be the endogenous origin of developing bone and cartilage[34-37]. This thus explains the relatively modest success of MSC-based therapies for the treatment of OA. Therefore, is there another population of stem cells that is more suited for stem cell-based therapy for OA?

Multiple articular cartilage stem/progenitor cell subsets have been discovered in recent years within the superficial zone of the articular cartilage[38-45]. They share similar characteristics including self-renewal capacity, multilineage differentiation potential in vitro, and the immunophenotypic expression profile of MSC-related surface markers[38,39]. Of particular interest to this study is the population of adult MSCs

with an increased affinity toward chondrogenic differentiation, marked by the genetic expression of Gremlin 1 (Grem1)[34]. These stem cells, named the Osteochondroreticular (OCR) stem cells, were shown to display a more restricted skeletogenic ability and do not give rise to adipocytes through both lineage tracing and in vitro differentiation, distinguishing them from the traditional MSCs[34]. Additionally, studies in mice demonstrated that the involvement of OCR stem cells was not restricted to development but further contributed to fracture repair in adulthood. An injury model of surgical fracture with internal fixation of the femur demonstrated that OCR stem cells contributed to bone and cartilage repair within the fracture callus[34]. Grem1 is a secreted bone morphogenic protein (BMP) antagonist that inhibits predominantly BMP2 and BMP4 in limb buds and is essential for limb bud development and digit formation[46-48]. However, less is known about the role of Gremlin 1 in skeletal development in adulthood.

The role of Grem1 in OA has been recently investigated using genetic knockout and surgically-induced OA models in mice (unpublished data). Grem1 is heavily involved in osteoblast differentiation and bone formation in adulthood with the loss of Grem1 expression contributing to an increase in bone mass[49]. Grem1 expression in articular cartilage, however, still remains to be elucidated. Deregulation of hypertrophic differentiation in articular cartilage has been shown to promote the onset and progression of OA[50,51]. Overexpression of Grem1 in the articular cartilage of mice inhibits hypertrophic differentiation of articular cartilage through Wnt-signalling and thus may present the key process to reversal of disease progression in OA[51]. Indeed, recent stem cell studies using in vivo lineage tracing to identify the true origin of skeletal tissues have shown that Grem1 expressing cells are abundantly present, not only in the articular cartilage, but also within the facet joint in mice[52]. Loss of BMP2 and BMP4 in OA articular cartilage further supports the role of Grem1 as an inhibitor of the BMP pathway[52]. Although Grem1 genetic expression seems to be essential for the delay of onset of OA pathology, intra-articular injection of Grem1 protein on the other hand exacerbates the disease[53]. With the contradicting evidence of the role of Grem1 at both the genetic and protein level, we set out to investigate the role of Grem1-expressing OCR stem cells in the onset and progression of OA. Using a transgenic lineage

tracing mouse model, this project aims to examine the role of Grem1-expressing stem cells in OA progression in comparison to the traditional MSCs.

3.4 Materials and Methods

3.4.1 Mouse Colonies

The following mouse lines were used in the study:

LepR-cre[54], *Acan-creER*^{T255}, *Grem1-creER*^{T34}, *R26-LSL-TdTomato*[56] are from Jackson Laboratory. All animal experiments were approved by the Animal Ethics Committee at the South Australia Health and Medical Research Institute (SAHMRI) under ethics number SAM189. The various sets of data described in the present study were gathered and verified across a minimum of 4 independent experiments.

3.4.2 Tamoxifen administration

Tamoxifen (#T5648, Sigma), for *creER*^{T} lines, was administered at 8 – 11 weeks of age. 4 x 6mg doses of tamoxifen dissolved in peanut oil were administered on different days within a week by oral gavage. DMM surgery was performed 1 week after the final dose of tamoxifen.

3.4.3 Histology

Bones were collected and fixed in 4% paraformaldehyde overnight, decalcified in Osteosoft® (#101728, Millipore) for 3 – 4 days and dehydrated in 30% sucrose at 4°C, embedded in OCT compound (Sakura Tissue-Tek) and kept at -80°C. 10µm frozen sections were collected on cryofilm (type IIC, Section-Lab) for staining. 0.04% toluidine blue (#198161, Sigma) in 0.1M sodium acetate pH4.0 and 0.1% fast green (#F7252, Sigma) in MilliQ water were used to determine cartilage and bone histology.

3.4.4 Osteoarthritis pathology scoring

A previously published scoring system[57,58] was modified to individually score structural damage and aggrecan loss.

Structural damage:

Score	Description
0	- **Normal Cartilage**
1	- **Roughened articular surface** - **Small fibrillations**
2	- **Fibrillation to immediately below the superficial layer** - **Some loss of surface lamina**
3	- **Horizontal cracks/separations between calcified and noncalcified cartilage** - **Or fibrillation/clefts to calcified cartilage but no loss of noncalcified cartilage**
4	- **Mild loss of noncalcified cartilage (<10% surface area)**
5	- **Moderate loss of noncalcified cartilage (10% - 50%)**
6	- **Severe loss of noncalcified cartilage (>50% surface area)**
7	- **Erosion of cartilage to subchondral bone**

Aggrecan loss:

Score	Description
0	- **Normal Cartilage**
1	- **Decreased but not complete loss of toluidine blue staining in noncalcified cartilage**
2	- **Focal loss of toluidine blue staining in noncalcified cartilage (<30% surface area)**
3	- **Diffuse loss of toluidine blue staining in noncalcified cartilage (>30% surface area)**

The mean of (a) the worst scores and (b) the sum of scores (sum of all sections in which a positive score was noted) from 2 observers blinded to genotype was calculated[59].

3.4.5 Immunohistological and fluorescent staining

Immunohistochemistry and immunofluorescent staining were completed on 10μm frozen sections prepared as above. Antigen retrieval was performed for immunohistochemistry by placing slides in a steamer submerged in antigen unmasking solution (#H-3300, VectorLab) for 6 min. Slides were treated

for endogenous peroxidase activity by incubating in 3% H_2O_2 for 30 min. Blocking was performed in 2% BSA, 5% normal goat and 5% normal donkey serum. Anti-PCNA (#ab18197 Abcam, 1:200) was used with incubation overnight at 4°C. Slides were then washed with PBST and incubated with Anti-rabbit biotin (#BA-1000, VectorLab 1:250) at room temperature for 1 h then streptavidin-HRP (#SA-5004, VectorLab 1:100) at room temperature for 30 min and developed with DAB chromogen (#K3468, Dako). Immunofluorescent slides were washed with PBST, permeabilised with 0.025% triton-X and counter stained with DAPI before mounted with cover slip. Lineage tracing and staining of slides were done on serial sections from pathology scoring slides.

3.4.6 Imaging

Brightfield images were obtained using the 3DHistech Pannoramic 250 Flash II. Fluorescent images were captured on the Olympus IX53 inverted microscope or the Leica TCS SP8X/MP confocal microscope.

3.4.7 Statistical Analysis

All analyses were performed using Prism 8 (GraphPad software Inc.).

3.5 Results

3.5.1 Surgical procedure

Destabilisation of the Medial Meniscotibial (DMM) surgery was performed on male mice between the ages of 10 – 13 weeks. Animals underwent general anaesthesia via isoflurane inhalation. The right hind limb of the animal was shaved with a razor blade to provide a clean surface. Left hind limb is used as a paired control (normal). Each end of the razor blade was used to shave a maximum of 5 animals to minimise razor burns. Animals were injected with sterile saline and ampicillin (50mg/kg) subcutaneously before a skin incision was made on the medial side of the patella, using sterile scissors, to expose the patella. Once the patella was exposed, a second incision was made on the medial side and the incision site was extended so that the patella can be luxated. Using sterile gauze, the fat pad located on the knee was pushed aside to expose the medial meniscotibial ligament, the ligament was then transacted using a twisting motion. Complete ligament transection was confirmed by the ability to manually displace the medial meniscus. Bleeding was controlled throughout surgery, and the cartilage was kept moist with sterile saline. Patella was then repositioned back, and the incision sites and the patella were sutured[59].

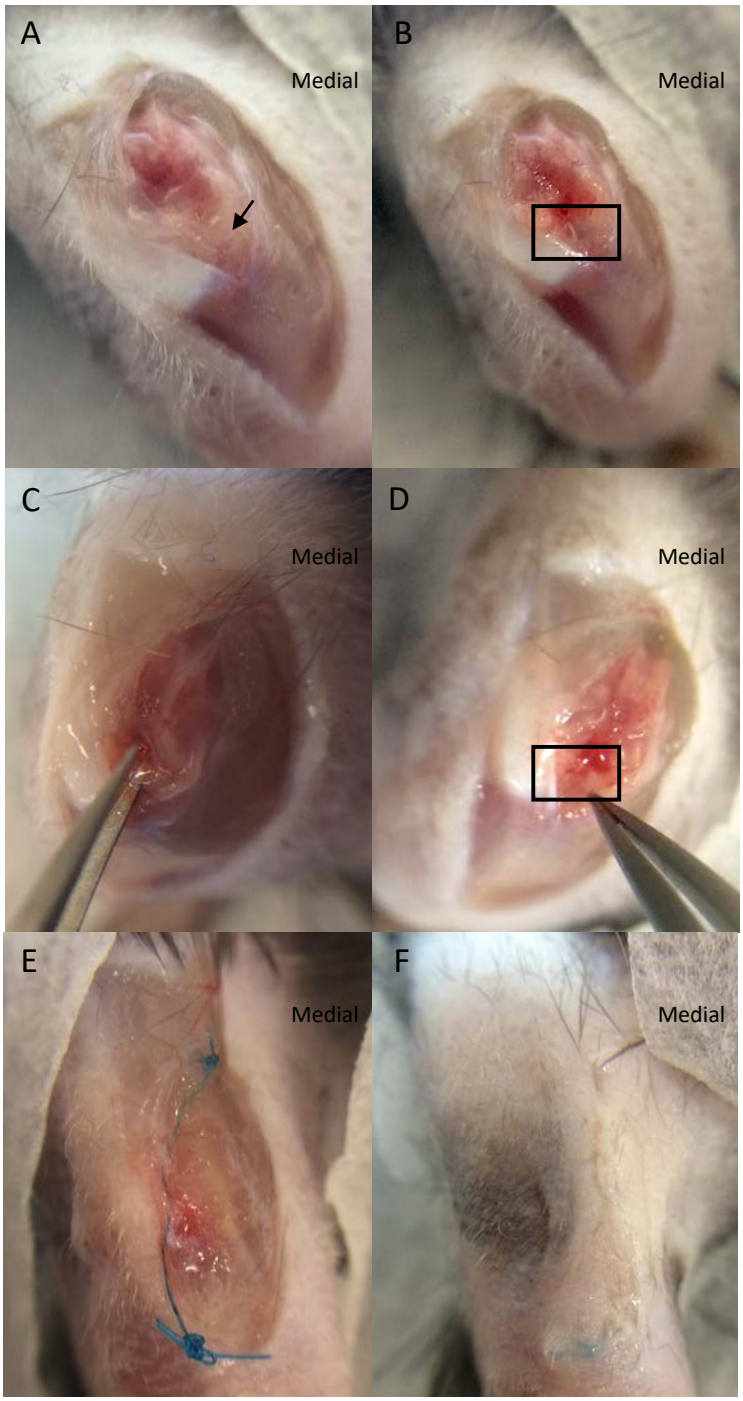


Figure 1. (A) Luxated patella exposing the fat pad (arrow) on the knee. (B) Exposed medial meniscus. (C) Sliding the curved tweezer under the meniscus to elevate the ligament. (D) Complete ligament transection confirmed by manually displacing the medial meniscus. (E) Sutured patella and incision sites. (F) Skin healed together by one suture under the skin and closed with tissue glue.

3.5.2 OA pathology identified and scored by histology staining.

Pathological changes of tissue affected by OA were identified by toluidine blue (cartilage) and fast green (bone) staining to indicate the proteoglycan integrity and osteophyte-like formation. At 2 weeks post-surgery (Figure 2C and D) the observed pathological changes are mild, indicative of the initial stages of OA. Proteoglycan loss is not as pronounced compared to normal (Figure 2A and B), demonstrated by the intensity change of purple staining (toluidine blue). However, early signs of OA can be seen by the fibrillation of the articular cartilage and synovial tissue inflammation. Surgical transection of the medial meniscotibial ligament is confirmed by the observed dislocation of the anterior meniscus. At this stage, initiation of an osteophyte-like growth can be noted on the anterior side of the tibia. At 8 weeks post-surgery (Figure 2E and F), features of progressive OA pathology become more prominent, with an evident loss of proteoglycans, indicated by the loss of toluidine blue staining, coupled with fibrillation in the articular cartilage and the persistent synovial tissue inflammation. At this stage, an osteophyte-like growth can also be seen, thus mimicking the OA pathology in humans. PCNA staining (Figure 2G - I) showed decreased proliferation of the articular chondrocytes across all three zones in the articular cartilage.

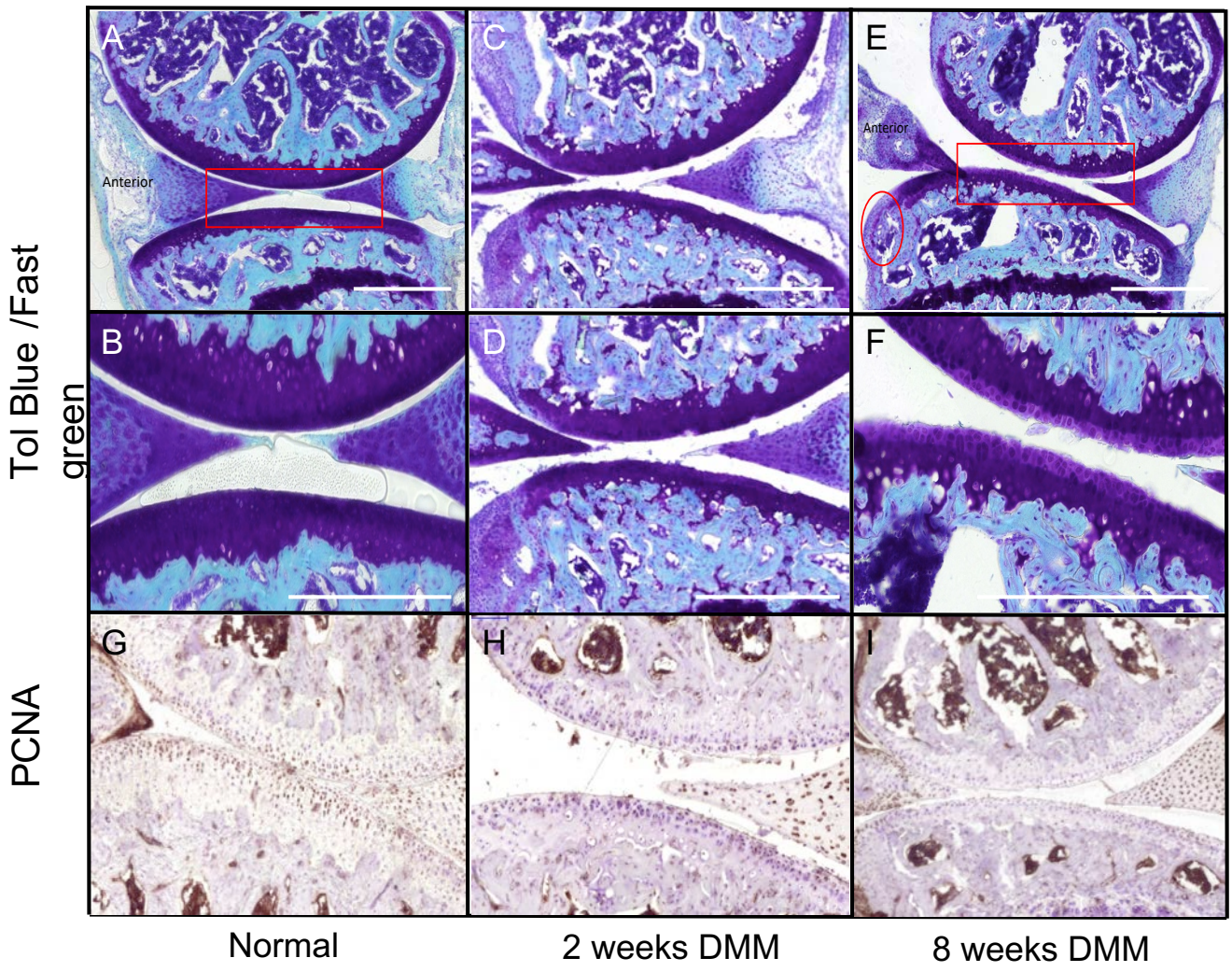


Figure 2. (A-F) Representative toluidine blue and fast green stain of normal (A-B), 2 weeks DMM (C-D) and 8 weeks DMM knees (E-F). (G-I) Representative PCNA stain for normal (G), 2 weeks DMM (H) and 8 weeks DMM (I) knees. (D-E). Scale bar = 500μm.

3.5.3 PCNA staining showed no evidence of repair or regenerate in early stage injury.

We hypothesised that articular chondrocytes would attempt to repair cartilaginous tissue during the early stages of OA. Further to this we assumed that this attempt, however, would eventually fail to comply with the increased tissue wear and tear and lead to the progression of articular cartilage destruction. To determine if articular chondrocytes could be induced to proliferate in an attempt to repair or regenerate tissue during the initial stages of OA, we decided to localise proliferating cell nuclear antigen (PCNA) in the DMM knees. Contrary to our hypothesis, our results indicate that articular chondrocyte proliferation was significantly decreased across all three zones of the articular cartilage (Figure 3A). Previously reported data of an increase in apoptotic articular chondrocytes in DMM-induced mice model of OA further supports our observation[60]. This trend of decreased articular chondrocyte proliferation continues with the progression of OA at 8 weeks post-surgery, with the exception of the calcified zone within the articular cartilage (Figure 3B). Additionally, the observed decrease in cell proliferation could be assigned to the process whereby chondrocytes undergo hypertrophic differentiation[61] thus, do not proliferate. This further justifies the lack of significant decrease in proliferation of the calcified zone of the articular cartilage at 8 weeks post-DMM, as chondrocytes in this zone are made up of hypertrophic chondrocytes. Comparison of the PCNA positive cells at 2 weeks and 8 weeks post-surgery, after normalisation to their paired control, showed no significant difference (Figure 3C). Our results further prove that articular chondrocytes do not attempt to repair or regenerate cartilage tissue through increased proliferation when challenged during OA.

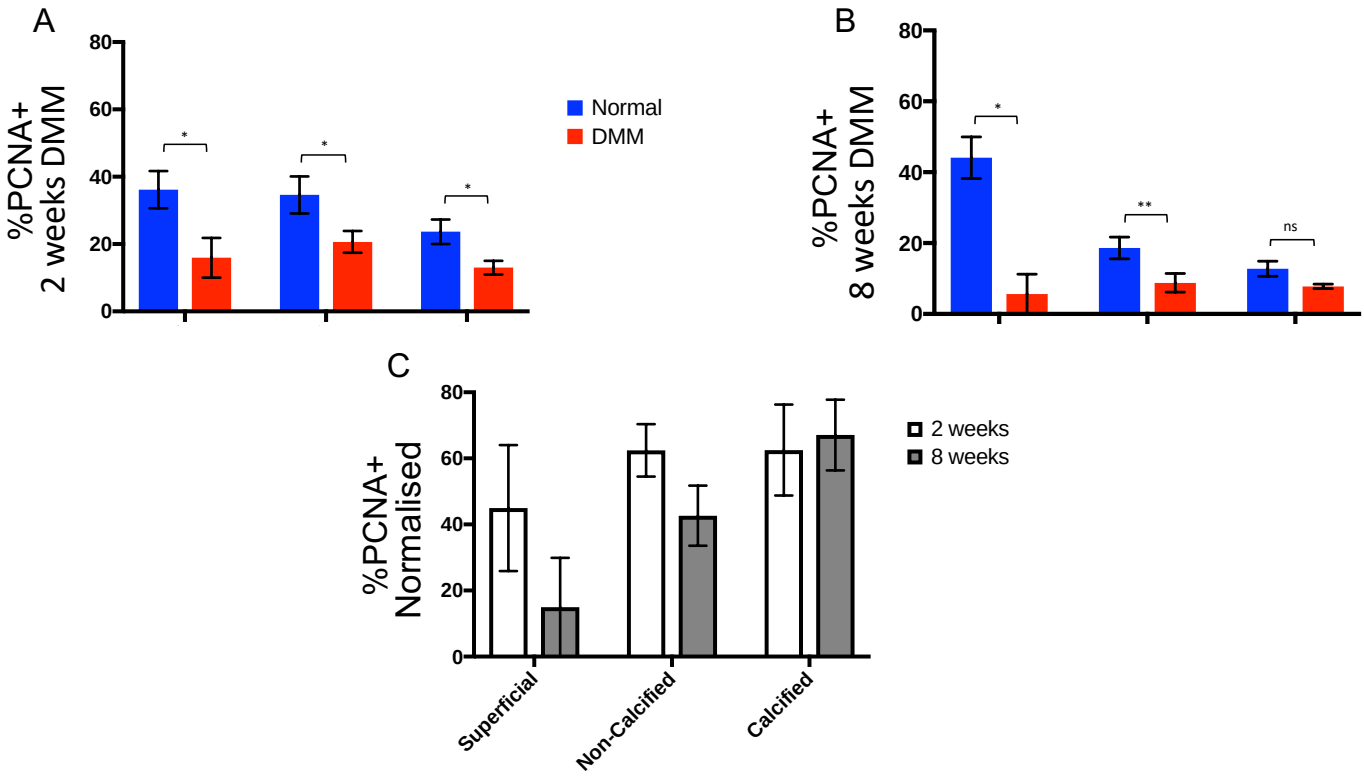


Figure 3. (A) Quantification of PCNA positive cells within the articular cartilage, 2 weeks post-DMM in all three individual zones of the articular cartilage. (B) Quantification of PCNA positive cells within the articular cartilage, 8 weeks post-DMM in all three individual zones of the articular cartilage. (C) Comparison of PCNA expression in cells of the articular cartilage, between 2 and 8 weeks post-DMM, normalised to their individual paired controls. All statistical analysis was performed on a minimum of 5 individual samples randomly selected with OA pathology verified by histological staining, but independent of pathology score.

3.5.4 Lineage tracing showed loss of Grem1-expressing articular cartilage cells in OA.

To study the role of the different skeletal stem cell populations in comparison to aggrecan expressing cells in articular cartilage during OA we utilised transgenic animals with fluorescent tags respectively driven by the genetic expression of Leptin receptor (LepR), Gremlin 1 (Grem1) and Aggrecan (Acan). LepR stem cells represent a well characterised, traditional skeletal perisinusoidal MSC population that resides within the bone marrow (Ding 2012, Zhou 2014). Grem1 marks a newly discovered skeletal stem cell subset that is distinct from the traditional MSCs as it does not give rise to adipocytes in vitro and in vivo. Grem1-expressing stem cells are of particular interest as they have been shown to give rise to articular cartilage in both developing and adult mice (unpublished data). As destruction of articular cartilage is central to initiation of OA, it is important to understand the role of Grem1 stem cells in articular cartilage during OA pathology. Acan is a well-defined extracellular matrix protein secreted by all cartilage cells during chondrogenic differentiation[62]. As previously reported, LepR stem cells gave rise to very rare articular chondrocytes throughout development and adulthood[35]. Thus, it is not surprising that LepR stem cells did not play a significant role in articular cartilage in OA. This finding supports the modest success in stem cell therapy for OA as most current stem cell therapies utilise MSCs extracted and expanded from bone marrow. During early OA (2 weeks post-DMM) Grem1 lineage traced animals, exhibited notable but not significant loss of Grem1-expressing articular chondrocytes compared to the paired normal controls. There was an observed increase in Grem1-expressing cell number in the anterior meniscus with an increase in proteoglycan secretion based on histological staining. A similar increase in numbers of Grem1-expressing cells, located in the anterior meniscus, can be observed in tissue samples collected 8 weeks post-DMM. At a later stage of OA, at 8 weeks post-DMM, the Grem1-expressing articular chondrocytes appeared almost completely diminished when compared to the paired normal controls. In comparison, Acan lineage traced articular chondrocytes did not demonstrate a decrease in number between their individual paired controls in both 2 weeks and 8 weeks post-DMM. This indicated that the loss of Grem1-expressing cells in the articular cartilage may be responsible for the progression of OA disease. The increase in the number of Grem1-expressing cells

may also be responsible for the increased proteoglycan secretion that helps in providing compressive resistance and shock absorbing capability of cartilage[63] as observed in the anterior meniscus.

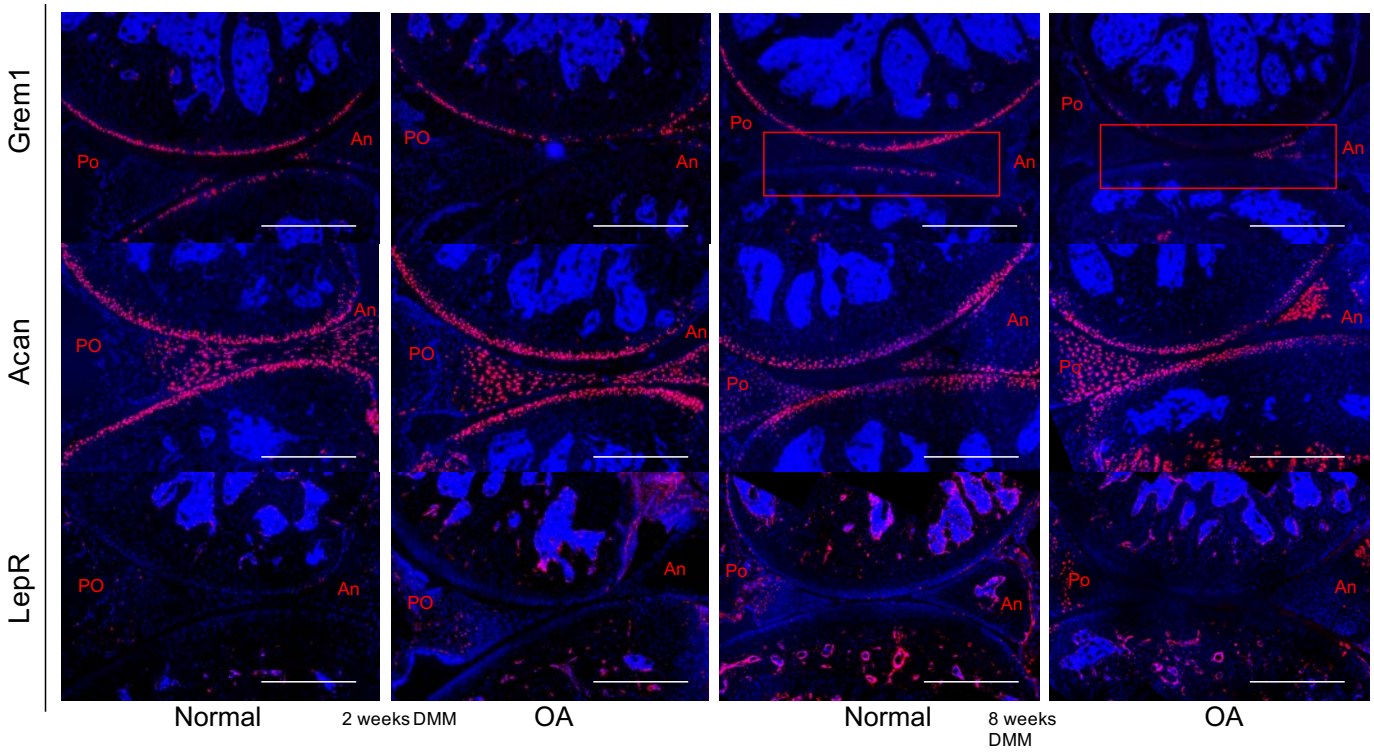


Figure 4. Representative images of Grem1, Acan and LepR lineage traced articular cartilage in the epiphyseal joint in 2 weeks and 8 weeks post-surgery, with their respective paired normal controls. A minimum of 5 independent paired samples were analysed for each population-traced cell. Scale bar = 500μm

3.5.5 Significant loss of Grem1-expressing articular cartilage cells only observed in later stages OA pathology.

By quantifying the number of cells traced by the expression of Grem1, Acan and LepR individually, we investigated the potential role of these cell populations and their contribution to the progression of OA. Lineage-traced population of cells (Grem1, Acan and LepR) were quantified as a percentage of DAPI positive cells in the superficial and non-calcified zones of the articular cartilage. The quantification area was determined by toluidine blue and fast green staining of serial sections, clearly outlining the areas of proteoglycan loss and injury. Our results showed that the total number of articular chondrocytes lost between the normal and OA knees was not significant in both, the 2 and 8 weeks post-DMM samples, indicated by the Acan-traced animals. As all chondrocytes express Acan, with majority of the cells from meniscus to the articular cartilage and growth plate being positive for Acan-TdTomato tracing, the Acan-lineage tracing was used as a control to elucidate articular chondrocyte biology during OA disease. This comparison demonstrated that the progression of OA pathology was not attributed to the loss of the total number of articular chondrocytes. A previous study reported that Grem1-expressing cells are located mainly in the superficial layer (top 2-3 cell layers) of the articular cartilage in adulthood, i.e. the layer most abundantly populated by articular chondroprogenitor cells[64-66]. Therefore, we examined the role of Grem1-expressing cells in the progression of OA pathology. Our findings indicate that the loss of articular chondrocyte at 2 weeks post-DMM was not significant compared to the paired normal control. By 8 weeks post-DMM (moderate stage of OA) however, the number of Grem1-expressing articular chondrocytes had significantly decreased particularly in the area of proteoglycan loss indicated by the toluidine blue staining. This implies that Grem1-expressing chondrocytes may be important for the progression of OA by holding a significant role in proteoglycan secretion, a process critical to maintaining articular cartilage integrity and protection from daily mechanical insult. Thus, the loss of Grem1-expressing articular cartilage cells may lead to the progression of OA disease. LepR cells on the other hand showed very little articular chondrocyte

tracing, therefore showed no significant loss of cells between paired normal and OA knees in both 2 and 8 weeks post-DMM.

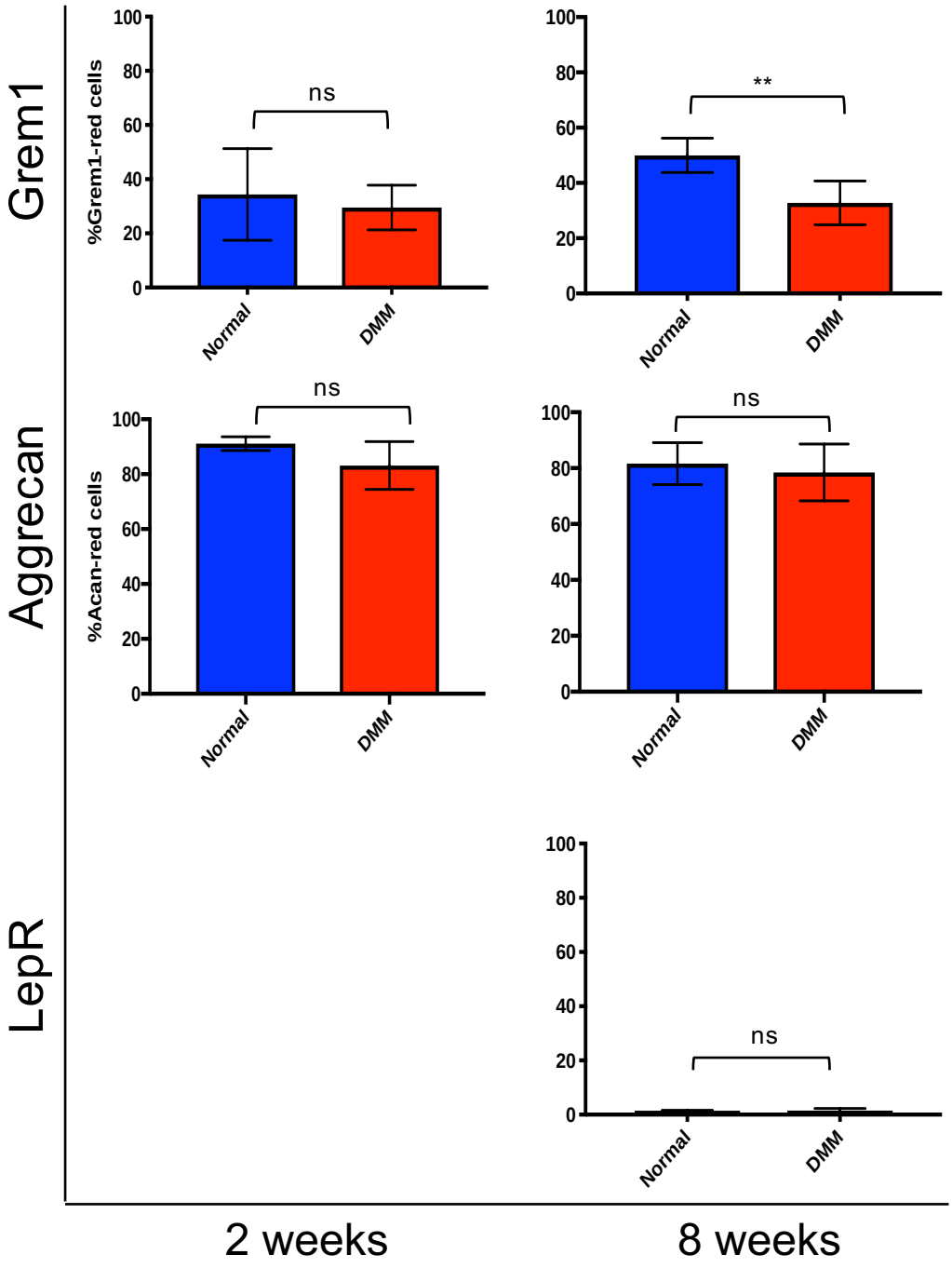


Figure 5. Quantitative graph of lineage traced Grem1-, Acan- and LepR-expressing cells in adulthood between paired normal and OA knees. Histological staining was performed on serial sections of all samples to confirm injury before images were selected for quantification. A minimum of n=4 independent paired samples were used for analysis.

2.5.6 LepR-expressing cells contributed to osteophyte-like formation in OA pathology.

Osteocalcin localisation identified LepR-expressing cell populations as pre-osteoblastic progenitor/stem cells that give rise to osteoblasts (unpublished data). As such, we investigated whether LepR-expressing cells hold a role in formation of osteophyte-like structures in OA pathology. OA samples with osteophyte-like formations were selected using toluidine blue and fast green staining, which identified newly generated bone, as a consequence of OA. Serial sections from these samples were used to trace LepR lineage cells within the osteophyte-like structures. LepR-lineage cell tracing at 8 weeks post-injury showed that LepR cells did not give rise to bone structure (negative for toluidine blue proteoglycan staining) within the osteophyte-like formations (Figure 6). However, LepR lineage cells gave rise to perivascular cells within the new subchondral vasculature as well as an insignificant number of chondrocytes (based on the morphology and size of cells) within the osteophyte. The process of endochondral ossification, whereby chondrocytes lay down the matrix for the invasion of osteoblasts, thus leading to bone formation and elongation has been well described. As such, we hypothesised that chondrocytes of the LepR lineage would hold a similar role in the formation of osteophytes. This assumption is further supported by the previously reported overlapping role of LepR-expressing cells and osterix-expressing cells, and osterix involvement in cartilage formation in embryogenesis as well as fracture injury[67].

LepR lineage cells appear to also contribute to the formation of the synovium lining, consistent with previously reported data (unpublished data). The synovium within the synovial capsule compartment holds a critical maintenance role by providing nourishment to the tissue and further by facilitating recruitment of inflammatory cells to the site of inflammation required for osteophyte-like formation in OA. Moreover, angiogenesis is fundamental to new tissue formation ensuring a consistent and continuous supply of nutrients in accordance with the increased demands of neogenesis. LepR is widely known to mark a population of haematopoietic stem cells located in the perisinusoidal space within the bone marrow, giving rise to multiple blood cell lineages and maintaining the stem cell niche within the

marrow[37]. In the case of OA and osteophyte-like formation, LepR-lineage cells were shown to give rise to perisinusoidal vascular cells forming the new subchondral marrow space within the osteophyte.

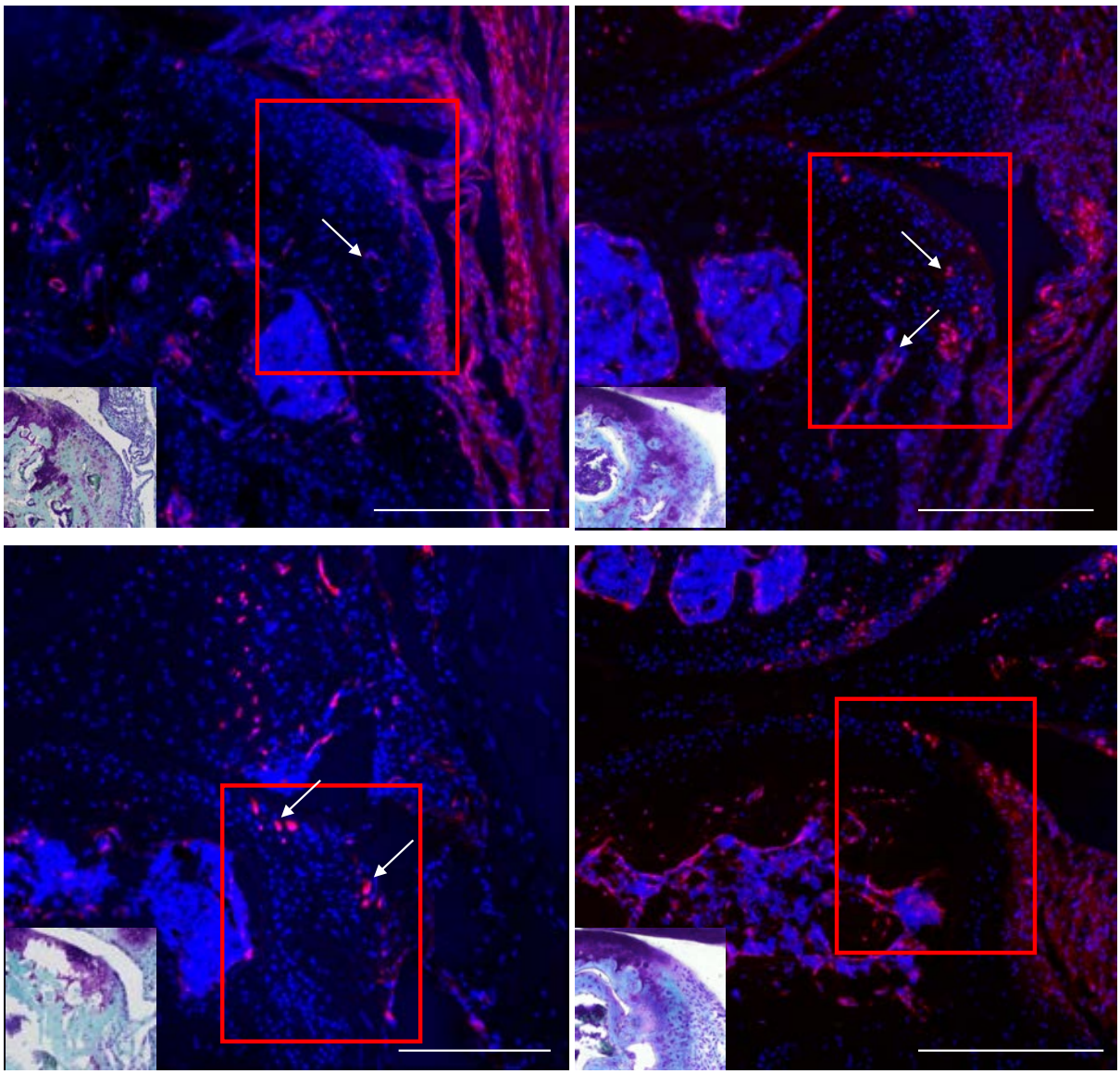


Figure 6: Images of the osteophyte-like formation in LepR lineage traced mice 8 weeks post-DMM surgery. Osteophyte samples were selected based on histological staining (Toluidine blue and Fast green) and serial sections were used for fluorescence imaging. 4 independent samples were selected for analysis. Scale bar = 300µm.

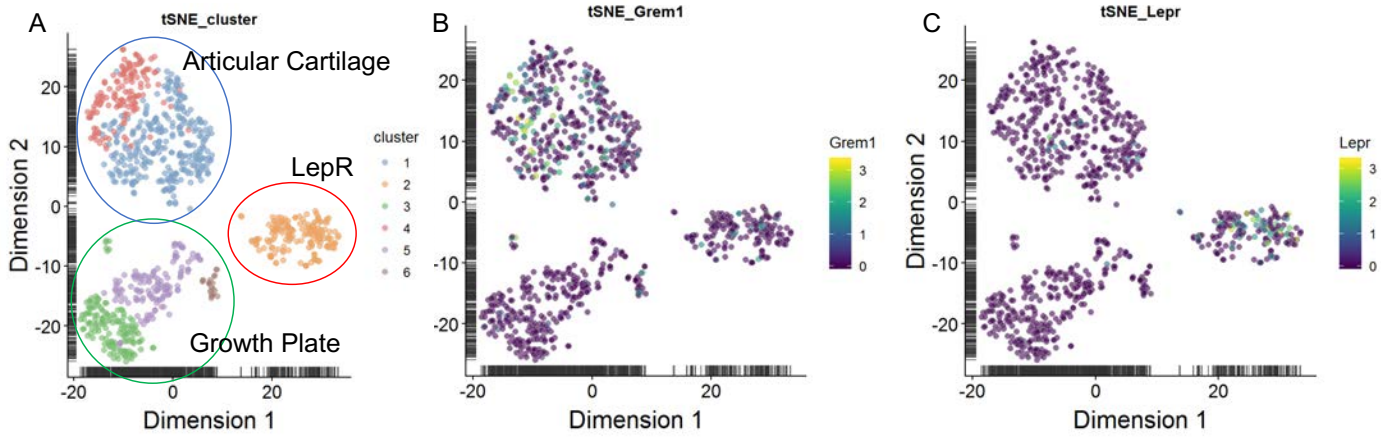
2.5.7 Grem1-expressing articular cells showed distinct genetic expression compared to growth plate and LepR-expressing cells.

To understand the different population of skeletal stem cells on a genetic level, we subsequently performed single-cell RNA sequencing (scRNA) on the cells isolated from lineage traced animals. Guided by our observations from histological slides of the lineage traced animals, cells were isolated from digested whole bones in from the LepR animals. While only articular cartilage and growth plate from the Grem1 lineage traced animals were mechanically detached from the bone under a dissection microscope and digested to achieve single cell suspension. Live single cell ($DAPI^-CD45^-Ter119^-CD31^-$ and marked by endogenous fluorescence) from the different population were then sorted into lysis buffer in a 96 well plate (1 cell per well) and sent out for scRNA preparation.

Our results showed distinct cluster of cells from the Grem1 articular cartilage and growth plate, as well as LepR cells (Figure 7A). The Grem1 expression cluster showed that the articular chondrocytes expressed higher levels of Grem1 expression (Figure 7B) than the ones isolated from the growth plate and that cells isolated from the LepR animals constituted majority of the LepR-expressing cells (Figure 7C). The heat map data showed that the Grem1-expressing articular chondrocytes expressed high levels of Prg4 (Proteoglycan 4/Lubricin), which is glycoprotein important for cartilage integrity[68] as well as a marker previously identified as articular cartilage progenitors[45]. Moreover, DCN (Decorin), a proteoglycan found primarily in the extracellular matrix of articular cartilage was also expressed exclusively in the Grem1 articular chondrocyte population. While both Grem1-expressing articular and growth plate chondrocytes expressed collagen type II which is a marker for hyaline cartilage. LepR cells on the other hand expressed Camp (Cathelicidin) and s100a9 (S100 calcium-binding protein A9) which are genes involved in the inflammatory response and myeloid cell dysfunction. Furthermore, LepR was also previously identified as a marker for haematopoietic stem cells (HSCs)[37] explaining their role of the LepR-expressing perivascular cells located in the bone marrow. Interestingly, other than

their well-established role as HSCs, LepR-lineage cells also expressed higher levels of Col1a2 (Collagen type 1 alpha 2), which is a marker for bone compared to the Grem1 cells.

The scRNA sequencing data concluded that Grem1-expressing cells are population of cells with more chondrogenic properties while LepR cells on top of their haematopoietic properties also possesses osteogenic characteristics.



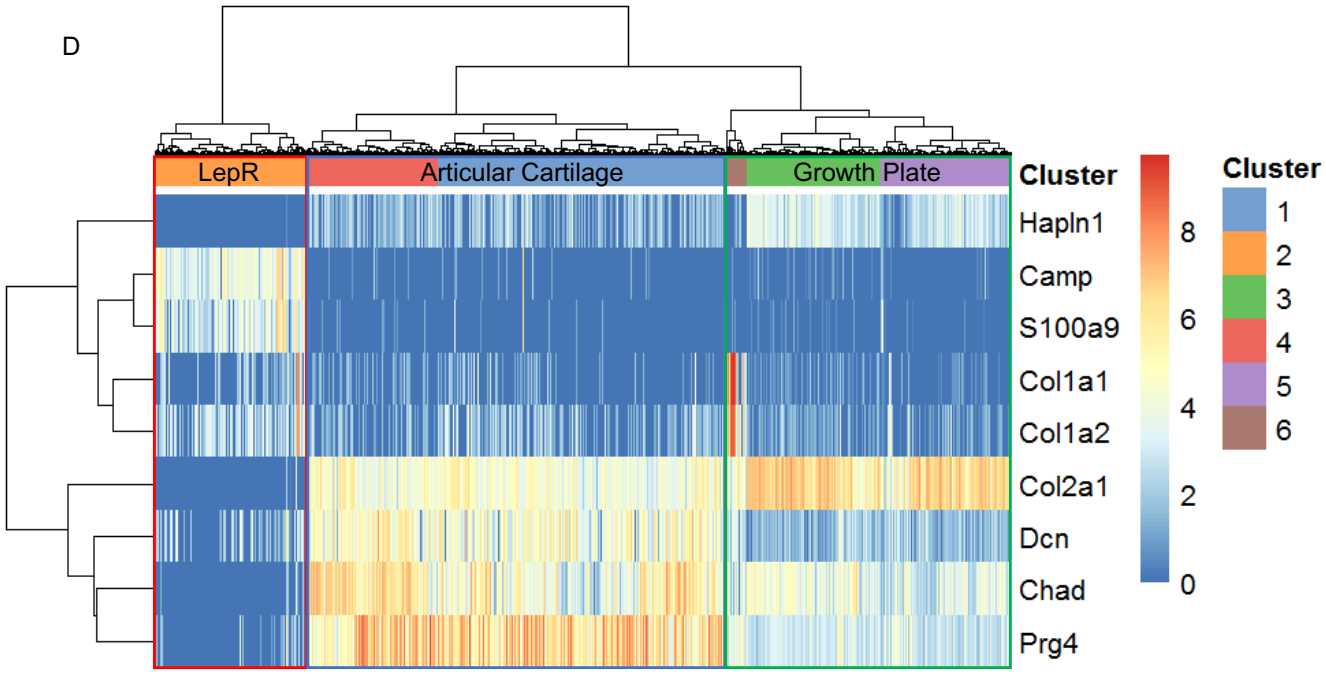


Figure 7. (A) Cluster map showing clustering of different population of cells based on differential expression. (B) Cluster map showing Grem1 expression within the same cluster groups. (C) Cluster map showing LepR expression within the same cluster groups. (D) Heat map of differentially expressed genes between the LepR-lineage cells, Grem1-lineage articular chondrocytes and Grem1-lineage growth plate chondrocytes.

2.6 Discussion

OA is a disease of the joints characterised by clinical symptoms associated with the degeneration of articular cartilage. Current treatments have displayed limited potential for preventing OA progression. As OA is initiated by the loss of a single cell lineage (articular cartilage) and often occurs within an easily accessible compartment for cell delivery, it presents as a potential candidate for application of regenerative stem cell therapy. In this study, we investigated and characterised previously identified skeletal stem cell populations and cartilage cells to understand their role in OA disease progression. Using a surgery-induced model of OA, transecting the medial meniscotibial ligament, dislocating the meniscus and creating a destabilisation of the knee in mice, tissue destruction was achieved through the natural wear and tear of joint movements, mimicking the pathology of OA observed in humans. This model of OA is widely used for OA related studies because of its reproducibility and the relatively short timeframe in which pathological changes become apparent. OA pathology was determined by histological staining with toluidine blue and fast green with effectiveness of surgery indicated by the fibrillation and loss of proteoglycan (purple stain) at the articular cartilage, a dislocated anterior meniscus as well as an osteophyte-like formation in the later stage disease.

Postnatal articular cartilage undergoes tremendous growth, modelling and remodelling. It is suggested that the proliferating cells in the top layer of the superficial zones are responsible for interstitial growth within the articular cartilage[69]. Due to the presence of stem/progenitor cells in the articular cartilage, we hypothesised that OA disease progression may result from the failure of articular stem/progenitor to keep up with the repair of degenerated articular chondrocytes initiated in the superficial zone. As such, we expected to see an increase in proliferating articular chondrocytes, particularly in the superficial zone during early stages of OA. However, our investigation, using PCNA staining, showed that there was no sign of regeneration as indicated by the lack of proliferation of articular chondrocytes. Even in the early stages of the disease where destruction was limited to just the superficial layer of the articular cartilage at 2 weeks, a significant decrease was noted in the number of proliferating articular

chondrocytes. This significant decrease continued into later stages of OA, suggesting that the loss of articular chondrocytes is key to the progression of OA disease. This is consistent with the fact that articular chondrocytes do not proliferate as part of tissue repair or regeneration in both early and later stages of disease. This result further indicate that the progression of OA disease was caused by the loss of proliferating articular chondrocytes. By 8 weeks post injury, there was no significant decrease of proliferating hypertrophic chondrocytes in the calcified zone of the articular cartilage. This observation could be attributed to the increase in hypertrophic chondrocytes that can be seen during OA progression.

OA is a degenerative disease that to date, has no consistently successful clinical treatment to halt its progression. Current stem cell therapies have shown limited capacity in terms of treating the long-term progression as well as sustaining the integrity of articular cartilage. From previous reports, traditional MSCs isolated from multiple sources such as bone marrow and adipose tissue, that had been commonly used in clinical treatment of OA, have resulted in inconsistent therapeutic outcomes[70]. Although MSCs have been shown to possess chondrogenic differentiation potential in vitro, we have reported that the bone marrow-derived MSCs marked by the expression of Leptin receptor (LepR) do not give rise to articular chondrocytes in development and rarely in adulthood in mice (unpublished data). This thus may explain the lack of efficacy of MSC based treatment in reversing progression of OA. Could the current stem cell therapy be using the wrong source of skeletal stem cells? In contrast, a newly discovered skeletal stem cell, marked by the expression of Grem1, has been shown to have chondrocytic differentiation potential in vitro and importantly, has further demonstrated to be highly involved in articular cartilage formation in early postnatal development as well as in adulthood (unpublished data).

In our study comparing both traditional MSC and Grem1-expressing skeletal stem cell populations to a common cartilage cell population, marked by the expression of Acan, we observed a significant loss of Grem1-expressing articular chondrocytes in later stage OA. This loss is consistent with the progressive loss of proteoglycan production indicated by the toluidine blue staining. Coincidentally, the anterior

meniscus, an area of increased proteoglycan production, traced the presence of a large number of Grem1-expressing cells during injury. This finding suggests that Grem1-expressing cells may be responsible for the production of proteoglycan. In addition, scRNA data also showed increased expression of Grem1 in the articular chondrocytes compared to the growth plate chondrocytes. These articular chondrocytes also expressed high levels of Prg4, which is a marker of articular cartilage progenitor cells[45] as well as DCN, which is primarily expressed in the extracellular matrix of articular cartilage[71]. These findings demonstrate that Grem1-lineage cells may have a role in maintaining articular cartilage integrity through the production of proteoglycan and the loss of such cells could drive the progression of OA. There was no significant loss of Acan-expressing cells noted, perhaps due to the large number of Acan-expressing cells present within articular cartilage. However, this observation demonstrates that Acan-expressing cells do not contribute to the progression of OA. Previous reports show that Acan-expressing stem/progenitors in the superficial zone may actually be the same population of Grem1-expressing articular cartilage stem/progenitors (unpublished data). Grem1-expression in the Acan-expressing clones in vitro is important in the propagation of the cells, therefore highlighting their significance in self-renewal stem/progenitor capabilities. In light of this, progression of OA disease could actually be attributed to the loss of Grem1-expressing stem/progenitor cells within the articular cartilage. This hypothesis is further supported by previous data showing that the Grem1-expressing articular chondrocytes are absent in knees of mice of old age. As primary OA is a disease of old age, the loss of Grem1-expressing cells may not only lead to the progression of injury-induced pathology but may also lead to spontaneous old age OA, further underlining the involvement of these cell in the relevant pathological processes.

Although cells of the LepR lineage, did not contribute to any articular cartilage chondrocytes, their role in maintaining haematopoietic stem cells as well as bone formation has been well documented in previous reports[35,37]. Osteophyte formation manifests during the late stages of OA in humans and requires generation of new bone and additional blood supply to support the structure. In our study, osteophyte-like formation was achieved using an injury-induced OA model in lineage traced mice. As

expected, LepR lineage cells can be seen to give rise to perivascular cells within the subchondral blood supply of the newly formed osteophyte-like structures, although further confirmation is required by co-localisation of CD31. The newly formed blood vessels may present a means to increase the blood supply and nutrient delivery required for osteophyte growth. Further to that, the LepR lineage cells gave rise to the synovium lining within the epiphyseal joint of the long bones. The synovium is a source of nutrients and the site of inflammatory cell recruitment during disease and injury. With synovitis being a common feature of OA, we speculate that the LepR cells may contribute to the recruitment of inflammatory cells in our study. This speculation was further supported by our scRNA sequencing data showing high expression levels of genes such as Camp and S100a9, that are known for their role in inflammatory cell recruitment. Inflammatory cells, in particular macrophages which have been shown to be the predominant inflammatory cells type in OA[72-74], home to the synovial lining and sublining[75-77]. They are known to aid in the progression of OA leading to cartilage degeneration and osteophyte formation[78,79] through cartilage matrix remodelling[80,81]. Macrophages release catabolic mediators such as matrix metalloproteinase (MMPs)[72] and transforming growth factor beta (TGF-β) that can induce osteophyte formation[79]. Further studies such as co-localisation of expression of macrophage cells markers (CD14, MHC II) is required to verify our hypothesis.

More interestingly, we also noted tracing of some LepR lineage chondrocytes within the osteophyte structure. It has been previously shown that macrophages express factors to induce chondrogenesis of mesenchyme cells to promote osteophyte formation[82]. These chondrocytes may also play a role in the process of endochondral ossification, required for bone growth. Furthermore, LepR lineage cells expressed higher levels of bone markers such as Col1a2, supportive of their role in osteophyte formation. The role of LepR lineage cells in the formation of osteophytes and their mechanistic involvement is an interesting finding that required further exploration but is beyond the scope of this project.

3.7 Conclusion

Human OA pathology in mice can be recapitulated by the destabilisation of the medial meniscotibial ligament (DMM) surgery. This injury-induced model of OA allows us to study the fate of the articular chondrocytes, specifically the Grem1 articular chondrocytes in the progression of OA disease. Our study highlighted that the loss of proliferating articular chondrocytes leads to the progression of OA pathology. More importantly, the loss of Grem1 articular stem/progenitor cells potentially leading to OA progression. This is the first step in demonstrating that OA may actually be a disease of the stem cells.

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Chapter 4: Loss of Gremlin 1 contributes to damage in articular cartilage which leads to the development of Osteoarthritis

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4.1 Abstract

Stem cell-based therapy for OA has been investigated for since 2002 with limited success due to the avascular nature of the articular tissue as well as the unsustainability of stem cell repair. Stem cells used for these studies are mostly isolated from the bone marrow or adipose tissue. The limitation of stem cell therapy has been attributed to multiple reasons, 1) the heterogeneity of the stem cell population isolated, 2) the loss of chondrogenicity during in vitro expansion, and 3) the fibrous cartilage repair does not meet the needs of normal joint movement that requires a highly specific hyaline cartilage tissue for smooth gliding and mechanical loading. Recent skeletal stem cell advances have indicated that the traditional MSC may not be the true origin of articular chondrocytes, which may explain the modest results of MSC therapy. Recently, our group has discovered that Grem1-expressing OCR stem cells play an important role in postnatal articular cartilage development. The loss of these cells in old age is postulated to be a major cause for OA disease progression in old age joints. Utilising transgenic mouse models, we have been able to show that these cells were significantly lost in OA using a surgical model of OA in mouse. This study aims to further validate the important role of Grem1-expressing articular cartilage in the progression of OA by utilising transgenic model of ablation.

4.2 Statement of Authorship

Statement of Authorship

Title of Paper	Contribution of Gremlin 1 expressing stem cells to cellular architecture and stem cell biology of articular cartilage in development and adulthood.
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Principal Author

Name of Principal Author (Candidate)	Jia Ng		
Contribution to the Paper	First author and main contributor. Concept design, literature search, review and formulation of primary draft, in addition to reviewing and incorporating co-author comments and suggestions.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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4.3 Introduction

Osteoarthritis (OA) is a common joint disorder that is characterised by the degeneration of articular cartilage leading to the loss of joint function and chronic pain[1,2]. Although the molecular mechanisms that lead to the progression of joint destruction have been extensively investigated, there is currently still no treatment to delay the progression of OA[3-16]. Whilst OA is most commonly initiated by old age, secondary causes such as injury and trauma can also instigate disease development[17]. Finding an efficient pharmaceutical therapy for OA to reverse or delay disease progression has presently fallen short of its growing demand.

Mesenchymal skeletal stem cells (MSCs) in adulthood exist in multiple tissues, most notably adipose and bone marrow tissues[18-26]. Presumed to be the origin of skeletal tissues, they can be isolated with relative ease and are expandable through standard culture techniques with reasonable simplicity. Often characterised by their fibroblastic shape, immunophenotype, trilineage differentiation potential and long-term self-renewal capabilities[27], MSCs have become a popular option for tissue repair and regeneration. More importantly, their immunomodulatory properties in combination with their paracrine activity and secretion of bioactive molecules, hold a pivotal role in stem cells regenerative capacity[28-30] in OA. Yet the overall benefit of MSC-based therapies for OA has yielded somewhat modest outcomes[31-35]. It was suggested that MSCs may not be the true endogenous origin of developing bone and cartilage, contradictory to their in vitro lineage repertoire. The fact that MSCs do not generate normal articular cartilage or contribute to normal osteochondral skeletogenesis[36-39] may explain their lack of therapeutic effect in reversing OA progression.

The recently discovered osteochondroreticular (OCR) stem cells, marked by the expression of Gremlin 1 (Grem1) have attracted much debate as to whether there exists a different stem/progenitor cell population with enhanced articular cartilage regenerative potential within the skeletal tissues[36]. In

parallel with Chan et al, this study independently discovered a different population of skeletal stem cell, distinct from the traditional MSCs, that gave rise to cartilage, but not fat, in animal models using in vivo lineage tracing[36,40]. Grem1 is a secreted protein that belongs to the tumour suppressor DAN family. It antagonises bone morphogenic protein (BMP) in the transforming growth factor β (TGFβ) signalling pathway[41]. Formally known to be down-regulated in mos-transformed cells (drm), Grem1 controls cell growth or viability and tissue-specific differentiation[42]. Grem1 is well known for its important role in guiding limb bud development and regulation of early development[43,44]. Since its discovery, Grem1 has been largely implicated in the inhibition of BMP 2 and BMP 4 signalling[45,46].

BMPs are a group of growth factors originally discovered for their ability to induce formation of bone and cartilage[47]. They are involved in the induction of endochondral ossification and chondrogenesis with particular effect in differentiating mesenchymal cells towards osteoblastic lineages[48]. BMPs also stimulate chondrocyte maturation and induce chondrocyte hypertrophy by increasing the expression of type X collagen and alkaline phosphatase[49,50] in these cells. With chondrocyte hypertrophy underling the pathogenesis of OA[51], it is not surprising that BMPs are involved in the progression of OA. The hypertrophic biochemical repair process is characteristic of early stage OA and induced by BMP-2 expression[52]. Initial degradation of the extracellular matrix (ECM) aggrecan is a response to create space in the articular cartilage for new tissue to be generated[53]. This degradation of the articular cartilage is caused by the upregulation of BMP-2 signalling which results in increased expression of downstream molecules such as Smad1 and Smad5, leading to chondrocyte hypertrophy[48]. In fact, BMP-2 and 4 are scarce in normal adult articular cartilage but are present in adult OA cartilage and osteophytes, specifically in the calcified zone of the articular cartilage in severe OA[54]. Although BMP-2 can be destructive for the progression of OA, BMP-2 overexpression can further have a contradicting effect on chondrocyte biology by increasing chondrocyte cell numbers and cartilage matrix production, leading to joint fusion[55]. Increased levels of BMP-2 protein associated with physiological exercise have also been shown to suppress post-traumatic OA progression[56]. It is therefore imperative that the

degradational properties induced by BMP not exceed the production of ECM for a reparative effect. With Grem1 being a BMP antagonist, one might speculate its pivotal role in maintaining this balance[53].

Despite the eminent role of Grem1 as a BMP antagonist, investigation into Grem1 involvement in OA is exiguous. Grem1, together with FRZB and DKK1, are prime candidates for delaying the progression of OA as all three inhibit chondrocyte hypertrophy in articular cartilage[57]. Chondrogenically-induced MSCs in hypoxic conditions such as that used to maintain and culture articular chondrocytes, have exhibited a robust increase in Grem1 expression[58]. Moreover, a mutation in Grem1 gene was reported to be associated with hip OA[57], and decreased in Grem1 expression was reported in degrading cartilage compared to healthy cartilage[59]. With OA being primarily a disease of old age, Grem1 expression was found to decline with an increase in age[60]. Yet, several comparative studies of human articular joints showed an increase in Grem1 expression in OA joints compared to normal[52,61]. Just like BMP-2 expression in OA, Grem1 expression can have contradicting effects depending on OA severity. Some studies have reported a downregulation of Grem1 in lower grades of OA, with expression localised within the middle and deep layers of mildly degenerated human cartilage[61-63]. It was further shown that expression of Grem1 increased steeply with the severity of OA, with highest concentrations detected in clustered chondrocytes in the superficial zone in severe human OA[61-63]. Others, in an injury-induced OA model, have shown elevated levels of Grem1 in the synovial fluid immediately following injury with a subsequent decrease with increasing time[60]. Nevertheless, the most recent study of Grem1 expression in OA showed a positive correlation between OA severity and Grem1 concentration. Again, in an injury-induced animal model of OA, significantly increased levels of Grem1 were detected even in early stages of OA. Intra-articular administration of Grem1 conjugated protein enhanced the severity of OA leading to a significant loss of proteoglycan in the articular cartilage[56,63]. The immense role of Grem1 in aggravating OA progression was further demonstrated by inducing OA with Grem1 injections alone in normal knee joints without injury. In addition, disease severity was reduced by intra-articular injection of Grem1 antibody. The role of Grem1 in OA was further substantiated using a conditional knockout of Grem1 in mice[63].

It is hard to discern the role of Grem1 in OA progression amidst the currently conflicting and limited evidence. In spite of our knowledge about the impact of Grem1 expression on OA pathology and progression, little is known about the cells that expresses Grem1 and their role in the disease. With the discovery of Grem1-expressing progenitor/stem cells in the articular cartilage in development and adulthood (unpublished data) and the current gap in therapeutic treatment to delay the progression of disease, this study intends to investigate the role of these cells and their contribution to OA disease progression. This study will be the first to uncover the implications of these cells and assess whether OA is truly a “wear and tear” disease or a disease of the stem cells.

4.4 Materials and Methods

4.4.1 Mice

We used the following lines: *Grem1-creER*^{T36}, *R26-LSL-TdTomato*^{64}, *R26-LSL-DTA*^{65}, *R26-LSL-iDTR*^{66}and *UBC-creER*^{T267} are from the Jackson Laboratory. *Grem1*$^{fl/fl}$ mice were a generous gift from Professor Simon Leedham, Nuffield Department of Medicine. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the Columbia University Medical Center (CUMC) under protocol number AC-AAAT1452 and AC-AAAR4433 or the Animal Ethics Committee at the South Australia Health and Medical Research Institute (SAHMRI) under ethics number SAM189. The various sets of data described in the present study were gathered and verified in a minimum of 3 independent experiments.

4.4.2 Tamoxifen administration

For *R26-LSL-DTA* experiments, animals were put on 500mg/kg tamoxifen chow diet at 8 – 13 weeks of age for continuous ablation of cells and collected 20 weeks after the start of tamoxifen administration. For *R26-LSL-iDTR* experiments, at 8 weeks of age, animals were either put on tamoxifen chow diet for 2 weeks before diphtheria toxin (DT) injections, or given 4 x 6mg doses of tamoxifen, dissolved in corn oil on different days within a week, by oral gavage, before administering DT injections 1 weeks after the last dose. All iDTR animals were given 300ng diphtheria toxin dissolved in PBS either via intraperitoneal (IP) injections only or both IP and right knee injections daily for 2 weeks. Animals were then sacrificed 48 hours after the last injection. For *UBC-Grem1*$^{fl/fl}$ experiments, 2mg of tamoxifen dissolved in peanut oil were administered once via intraperitoneal injection at Day 4 – 6 of age and subsequently 6mg of tamoxifen dissolved in peanut oil administered via oral gavage 3 times weekly.

4.4.3 Histology

Bones were collected and fixed in 4% paraformaldehyde overnight, decalcified in Osteosoft® (#101728, Millipore) for 3 – 4 days and dehydrated in 30% sucrose at 4°C before embedding in OCT compound (Sakura Tissue-Tek) frozen. 10μm frozen sections were collected on cryofilm (type IIC, Section-Lab) for staining. 0.04% toluidine blue (#198161, Sigma) in 0.1M sodium acetate pH4.0 and 0.1% fast green (#F7252, Sigma) in MilliQ water were used to determine cartilage and bone histology.

4.4.4 Immunofluorescent staining

Immunofluorescent staining was completed on 10μm frozen sections prepared as above. Antigen retrieval was performed by placing slides in a steamer submerged in antigen unmasking solution (#H-3300, VectorLab) for 6 min. Blocking was performed in 2% BSA, 5% normal goat and 5% normal donkey serum. Anti-PCNA (#ab18197 Abcam, 1:200) was used with incubation overnight at 4°C. Slides were then washed with PBST and incubated with Anti-rabbit biotin (#BA-1000, VectorLab 1:250) at room temperature for 1 h, followed by streptavidin-Alexa Fluor 647 (#S32357, Life Tech 1:200) incubation at room temperature for 30 min and counter stained with DAPI before mounting with cover slip. Lineage tracing slides were washed with PBST, permeabilised with 0.025% triton-X and counter stained with DAPI before mounted with cover slips. Staining of slides for lineage tracing was done on serial sections.

4.4.5 RNA isolation and RT-PCR

Bones from the front limbs of *UBC-Grem1*$^{fl/fl}$ animals were taken, gently disrupted using a mortar and pestle, and digested in 2.5mg of collagenase I (#CLS-1, Worthington) at 37°C for 3 hours. Cells were then filtered through a 40um filter and red cells lysed using ACK lysis buffer (#A1049201, Gibco). Remaining cells were then collected for quantitative real-time PCR. Total RNA was isolated from cells using TRIzol (#15596018, Invitrogen) as per manufacturer's instruction. Complementary DNA (cDNA) was generated using SuperScript IV reverse transcriptase kit (#18091050, Invitrogen)

according to manufacturer's protocol. Transcript levels were assessed by QuantStudio 7 (Thermofisher) using IDT probes GAPDH (Mm.PT.39a.1) and Grem1 (Mm.PT.53a.31803129).

4.4.6 *In situ* hybridization (ISH)

ISH analyses were performed on formalin-fixed and paraffin-embedded mouse tissue samples using RNAscope 2.5 HD Detection kit technology (Advanced Cell Diagnostics) according to manufacturer's instructions. Briefly, tissue sections were baked in a dry oven at 60°C for 1 hour and deparaffinised, followed by hydrogen peroxide blocking for 10 min at room temperature. Antigen retrieval was performed by boiling in target retrieval solution for 15 min and then incubation with the protease solution for 30 min at 40°C. Sections were then incubated with a mouse Grem1 probe: NM_011824.4, region 398 – 1359 (#314741, Advanced Cell Diagnostics) for 2 hours at 40°C, followed by successive incubations with Amp 1 – 6 reagents. Staining was finally visualised with DAB, followed by counterstaining with haematoxylin.

4.4.7 Imaging

Fluorescent images were taken either on the Olympus IX53 inverted microscope or the Leica TCS SP8X/MP confocal microscope.

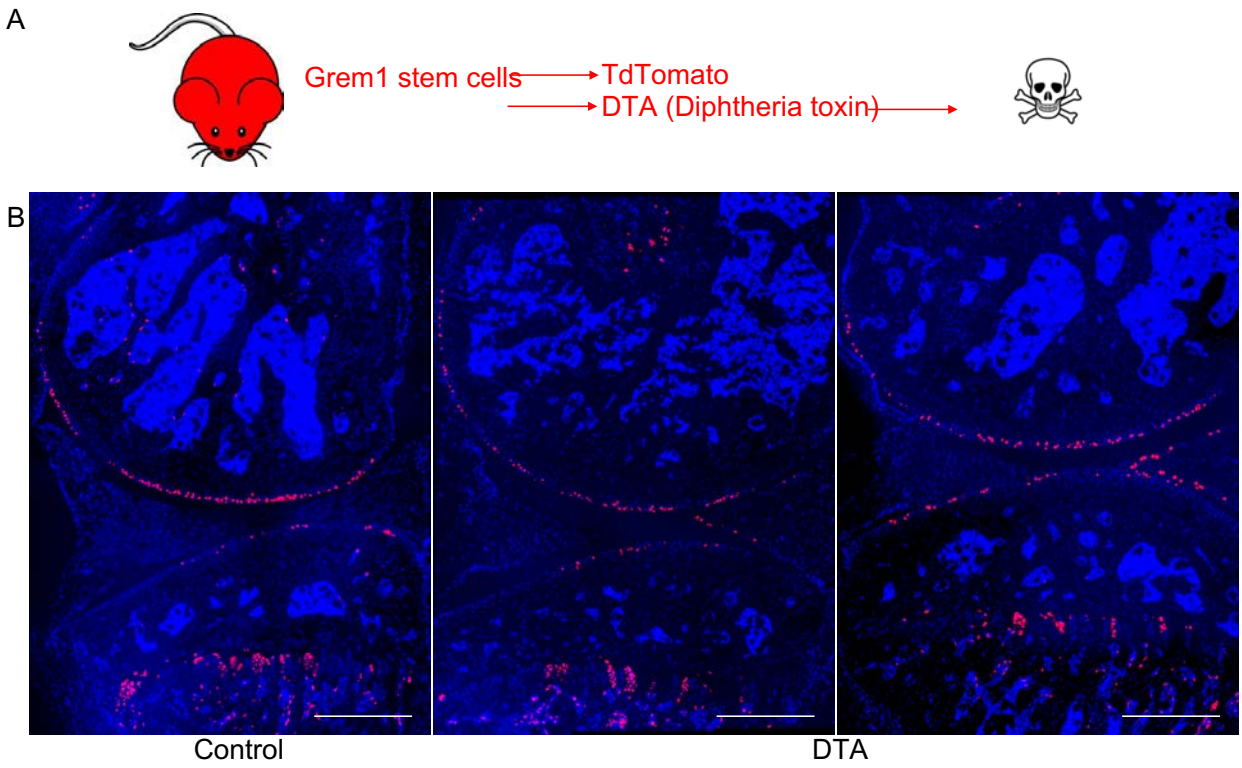
4.4.8 Statistical Analysis

All analyses were performed using Prism 8 (GraphPad software Inc.).

4.5 Results

4.5.1 Grem1-DTA model of ablation did not ablate a significant number of Grem1-expressing cells.

In order to validate the functional importance of Grem1-expressing cells in articular cartilage and in OA, we attempted to ablate the cells in transgenic animals to show that, without surgical challenge, the loss of Grem1 cells leads to an OA phenotype. The transgenic strategy was to test whether heterozygous (single dose) Cre in diphtheria-related strategies could effectively delete Grem1-expressing cells in articular cartilage. To achieve this, we used the diphtherial toxin fragment A (DTA) mice with a fluorescent tag (TdTomato) mated to Grem1-creERT. Upon tamoxifen administration, Grem1-expressing cells expressed the TdTomato fluorescent tag as well as the diphtheria toxin. Thus, theoretically, all Grem1-expressing cells should have been ablated, and the number of TdTomato positive cells quantifiable in the articular cartilage. Grem1-TdTomato-DTA mice were given tamoxifen chow to ensure a continuous ablation for up to 3 months starting at 8 weeks of age. Grem1-TdTomato littermates were used as controls. Our results indicate that this model of ablation was not successful as there were no observable difference between the controls and the DTA animals. Quantification, done on the limited samples, showed minimal change in the number of TdTomato positive cells. We were unable to do a statistical comparison due to the low number of control samples.



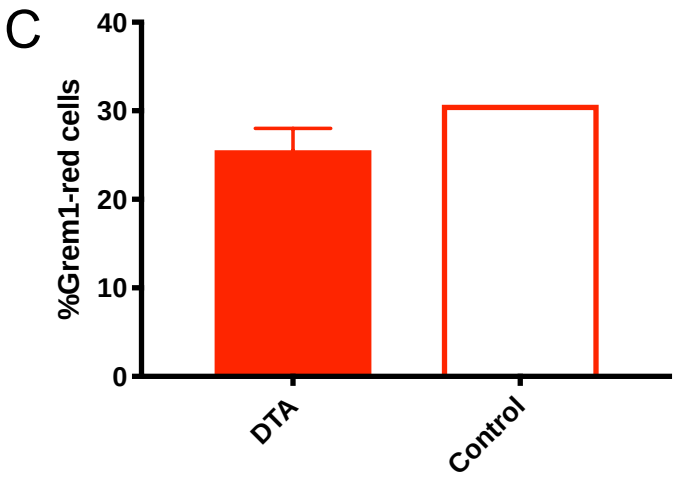
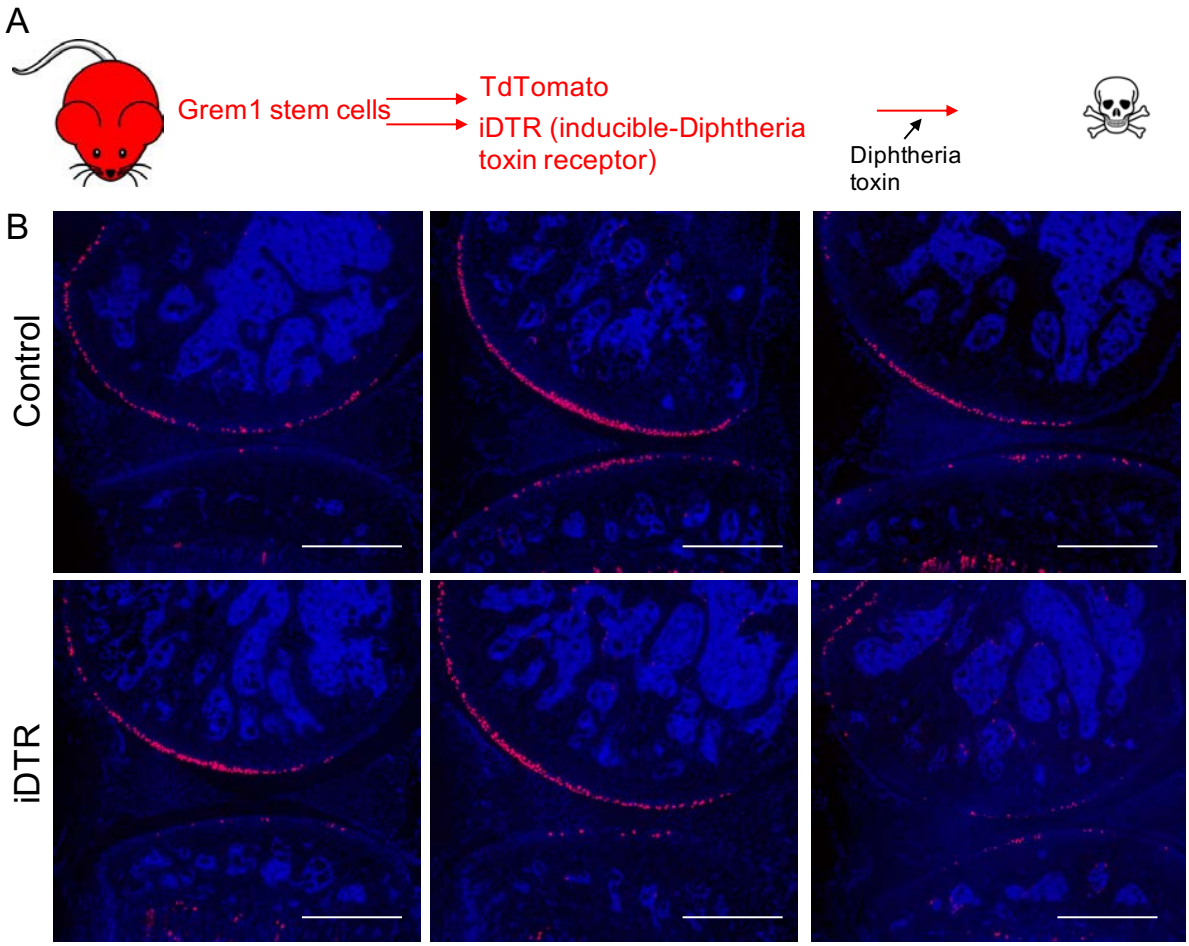


Figure 1. (A) Illustration of the transgenic mouse model of targeted ablation of Grem1-expressing cells. (B) Representative photos of transgenic animal knees that were given tamoxifen chow for 3 months. Control (Grem1-TdTomato) and DTA (Grem1-TdTomato-DTA). (C) Quantification of TdTomato positive cells in DTA and control mouse samples.

4.5.2 Grem1-iDTR model of ablation via tamoxifen chow does not ablate of Grem1-expressing cells consistently.

We next tested another model of ablation using the inducible diphtheria toxin receptor (iDTR) expression along with the fluorescent tag (TdTomato). In this model, an injection of diphtheria toxin (DT) was required to induce ablation. Mice were given tamoxifen chow at 8 weeks of age and DT via intraperitoneal injections 2 weeks after the start of tamoxifen chow. Grem1-TdTomato only littermates were used as controls. Ablation was quantified by the counting the total number of TdTomato positive cells as a proportion of DAPI positive cells in the superficial and non-calcified zones of the articular cartilage. Quantification showed that there was no significant decrease in the percentage of Grem1-postive cells between the control and the iDTR animals. Thus, indicating that Grem1-expressing cells were not successfully ablated. Since mice were left on tamoxifen chow throughout the treatment, we hypothesised that articular chondrocytes undergo a compensatory mechanism of dedifferentiation and proliferation to replace the ablated Grem1-expressing progenitors, thus leading to the lack of significant ablation observed. Therefore, we stained serial sections of the knees for PCNA to determine if an increase in proliferation could explain the unsuccessful ablation of the Grem1-expressing articular chondrocytes. PCNA staining showed that there was no significant increase in the total number of proliferating articular chondrocytes, although there was a notable increase in the mean percentage. This increase, however, was not specific to the Grem1-expressing articular chondrocytes. As TdTomato and iDTR expression in the animals were directly attributed to the amount of tamoxifen chow ingested by each individual animal, and comparisons were performed between independent animals left on tamoxifen chow, we wondered if the variable tamoxifen intake by each individual animal further contributed to the lack of consistent tracing and ablation. Considering the above-mentioned inconsistencies, we hypothesise that the unsuccessful ablation of the Grem1-expressing cells could be attributed to the inconsistent intake of tamoxifen chow by the individual animals and the increase in proliferation of articular chondrocytes while being left on tamoxifen chow during injections.


A
Grem1 stem cells
TdTomato
iDTR (inducible-Diphtheria toxin receptor)
Diphtheria toxin
B
Control
iDTR

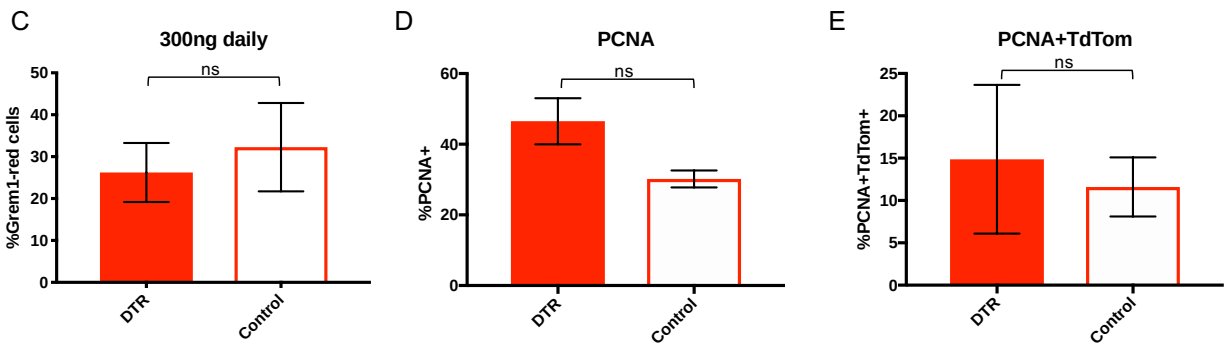


Figure 2. (A) Illustration of the transgenic mouse model of targeted ablation of Grem1-expressing cells. (B) Knee sections of an ablation model of Grem1-expressing cell specific transgenic mice, on tamoxifen chow at 12 weeks of age. (C) Quantification of the Grem1-expressing articular chondrocytes as a comparison of control and iDTR animals on tamoxifen chow with injections. (D) Quantification of total PCNA positive cells, and (E) percentage of Grem1-expressing proliferating articular chondrocytes in the superficial and non-calcified zones of the articular cartilage between control and iDTR animals. All statistical analysis was done using a minimum of 3 independent samples. Scale bar = 500µm.

4.5.3 Grem1-iDTR model of ablation via oral gavage with knee injections did not show significant ablation of Grem1-expressing cells.

Previously, having the iDTR animals on tamoxifen chow proved to be highly variable due to the inconsistent intake of tamoxifen chow between the control and experimental mice. We therefore decided to proceed our experiment with giving discreet doses of tamoxifen via oral gavage at 8 weeks of age and starting injections 1 week after the last dose of tamoxifen. With the iDTR model, we were able to ablate Grem1-expressing cells specifically located in the knee rather than systemically ablating of these cells. This was achieved by administration of DT into the synovial capsule via intra-articular injections. Due to the avascular nature of the articular cartilage, we believe that intraperitoneal injections of DT may not have been able to reach the articular chondrocytes and thus, unable to successfully ablate the cells. To counter the above variables, we also injected DT into the right knees of the mice on top of the intraperitoneal injection, leaving the left knees as paired controls. Mice without the iDTR expression were used as controls (Grem1-TdTomato). Lineage traced knees from controls as well as the iDTR animals did not show any observable differences. Total number of Grem1-expressing articular chondrocytes within the superficial and non-calcified zones was quantified as a percentage of DAPI. Quantification of the total Grem1-expressing articular chondrocytes confirmed our observations of no significant decrease in percentage of cells, indicating that the ablation was not achieved. A comparison between the paired knee samples also showed no significant decrease in Grem1-expressing articular chondrocytes, suggesting that the unsuccessful ablation was not attributed to the avascular nature of the tissue. All the above attempts of the transgenic mouse model of iDTR ablation demonstrated that the unsuccessful ablation of Grem1-expressing articular chondrocytes was not associated with the variables caused by inconsistent tamoxifen chow intake between independent animals as well as the injection strategies.

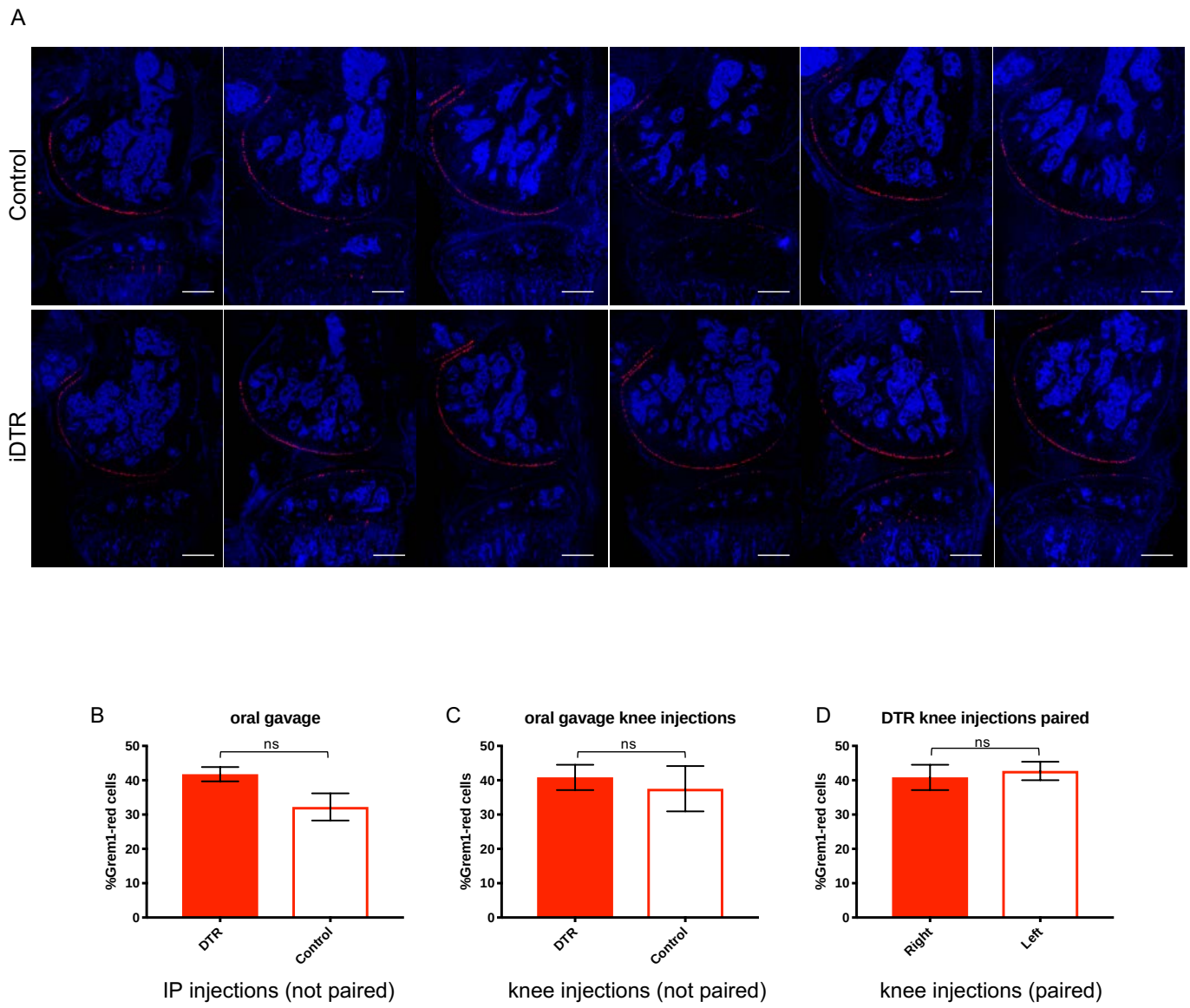


Figure 3. (A) Knee sections of the ablation model of Grem1-expressing cell specific transgenic mice, by oral gavage dosing. Knees were collected at 12 weeks of age. (B) Quantification of Grem1-expressing articular chondrocyte ablation via intraperitoneal injections, (C) intra-articular injections between independent animals and (D) intra-articular injections between paired knees within the articular cartilage. All statistical analysis was done with a minimum of 3 independent samples. Scale bar = 500μm.

4.5.4 A new knock-in transgenic model of ablation.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)- gene editing technology[68], allows for a faster knock-in transgenic animal model than previously described[69]. The DTR and TdTomato construct was designed to be inserted into the end of the coding region of exon 2 in the Grem1 allele, truncated with the 2A peptide protein. The 2A peptide protein unlike the traditional IRES does not have the risk of reducing protein expression as protein transcription is driven by only one promoter[70]. Post-transcriptional modifications cleave at the 2A site then allow for the expression of individual protein instead of the large fusion protein. This is particularly useful for Grem1 because of the low expression in adult tissues. Therefore, the slightest disruption of protein expression would alter the effect of DTR ablation. Using a guide RNA, this construct is delivered to the homology arm of Grem1 endogenous sequence and insertion through homologous recombination.

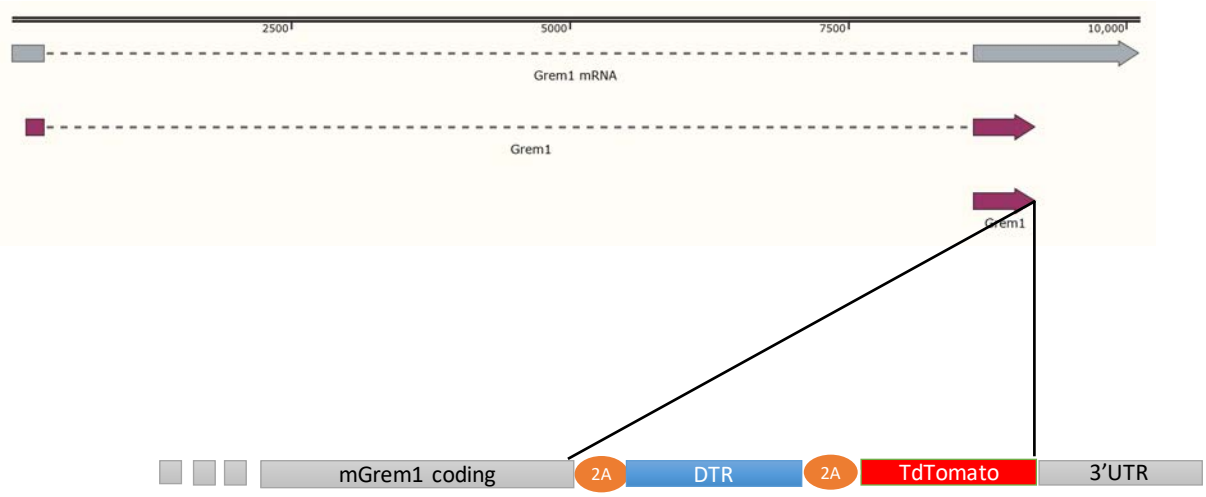


Figure 4. Diagram illustration of the genetic design of the genetically modified mice using CRISPR-gene editing technology.

4.5.5 Impact of Grem1 expression in skeletal development.

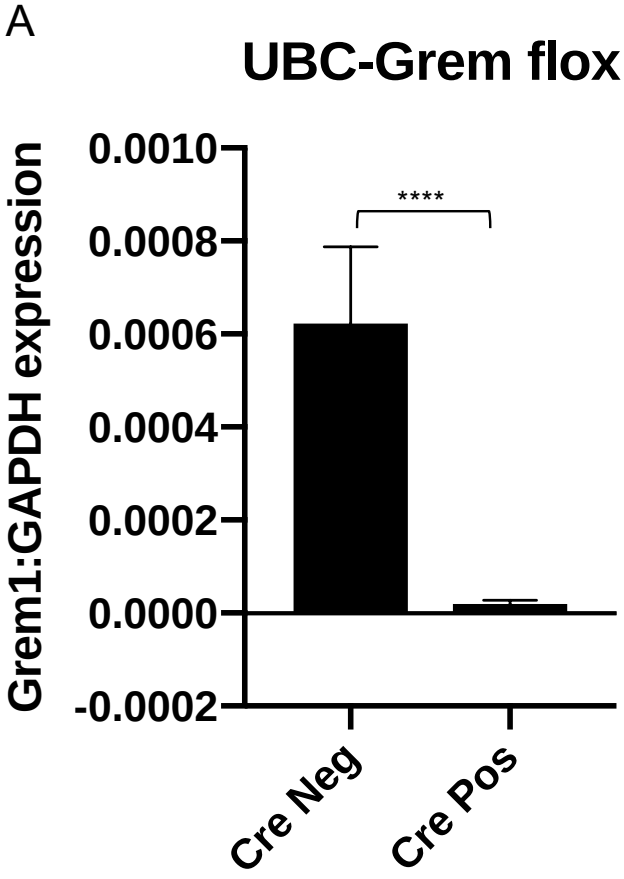
Grem1 expression marks the population of articular cartilage OCR cells, previously identified as an articular stem/progenitor cell subset (unpublished data). Since Grem1 expression plays an important role in defining these cells, we wondered if Grem1 expression is a significant factor in the maintenance and health of articular cartilage in development and adulthood. To do this we used animals with a ubiquitous knock-out of Grem1 expression induced by tamoxifen administration (UBC-Cre^{ERT}; Grem1$^{fl/fl}$), thereby known as UBC-Grem1 flox. UBC-Cre^{ERT} negative; Grem1$^{fl/fl}$ littermates were used as controls in this experiment. We know that Grem1-expressing OCR cells have a key role in the postnatal development of articular cartilage, hence we decided to knockout Grem1 expression during postnatal development to maximise the impact on the articular cartilage structure and integrity. To knockout Grem1, UBC-Grem1 flox animals were given one dose of tamoxifen at day 4 – 6 of age via intraperitoneal injection and then again 3 times weekly via oral gavage from 4 weeks of age.

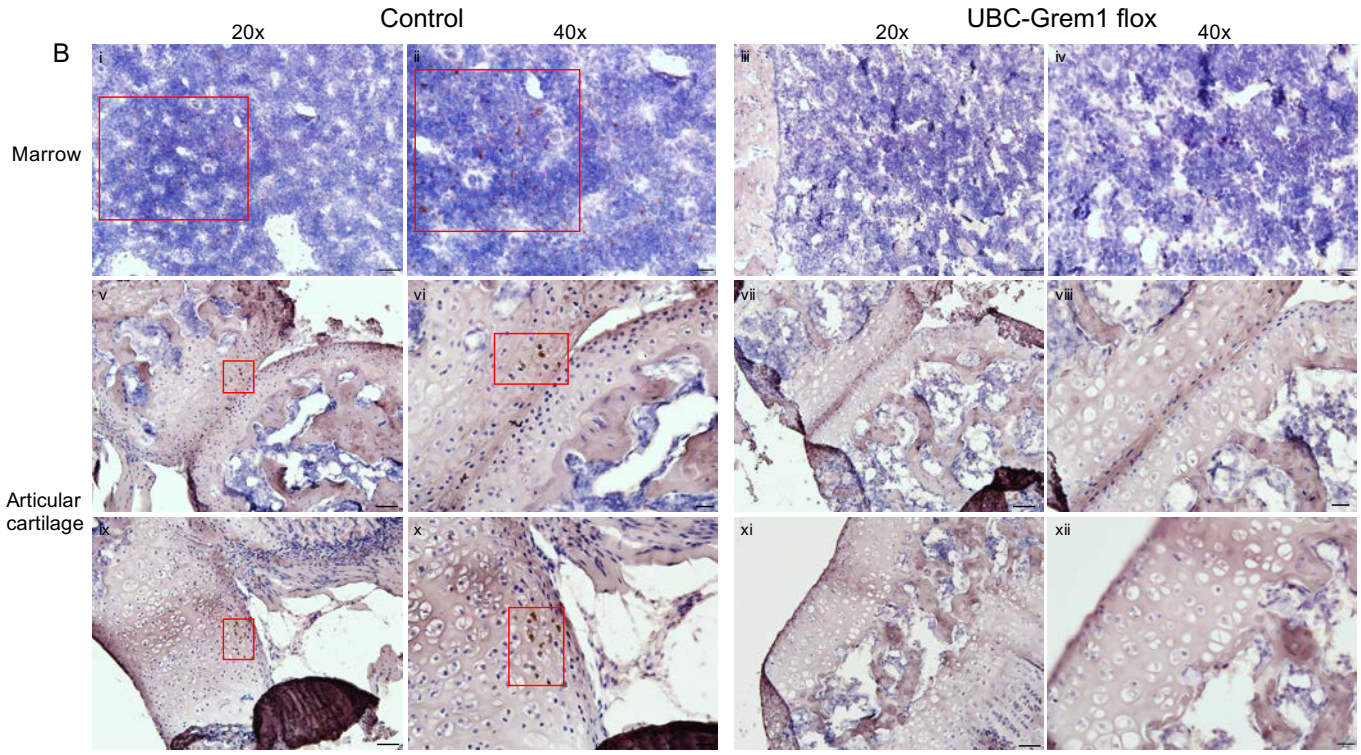
To validate the knockdown of Grem1 expression, we extracted RNA from digested whole bones isolated from the front limbs. qPCR was used to determine the level of Grem1 expression in the control and experimental groups. Results showed a significant knockdown in Grem1 expression (Figure 5A) indicating that the tamoxifen dosage was sufficient in our animal model. To further validate the knockdown in expression in the articular cartilage tissues, ISH (Figure 5B) was also used to stain for Grem1 expression in the bone marrow (i - iv) and articular chondrocytes (v – xii) within the epiphyseal joint of the hind limbs. These experiments demonstrated localisation of Grem1 in the control samples but did not show Grem1 expression in the UBC-Grem1 flox tissues. Although positive staining was noted in the control tissues, expression within the articular chondrocytes was minimal suggesting that Grem1 expression is not highly expressed in the articular cartilage of these mice. Grem1-positive chondrocytes were mainly localised within the intermediate zone of cartilage consistent with a previous report of gremlin expression in healthy human cartilage[52].

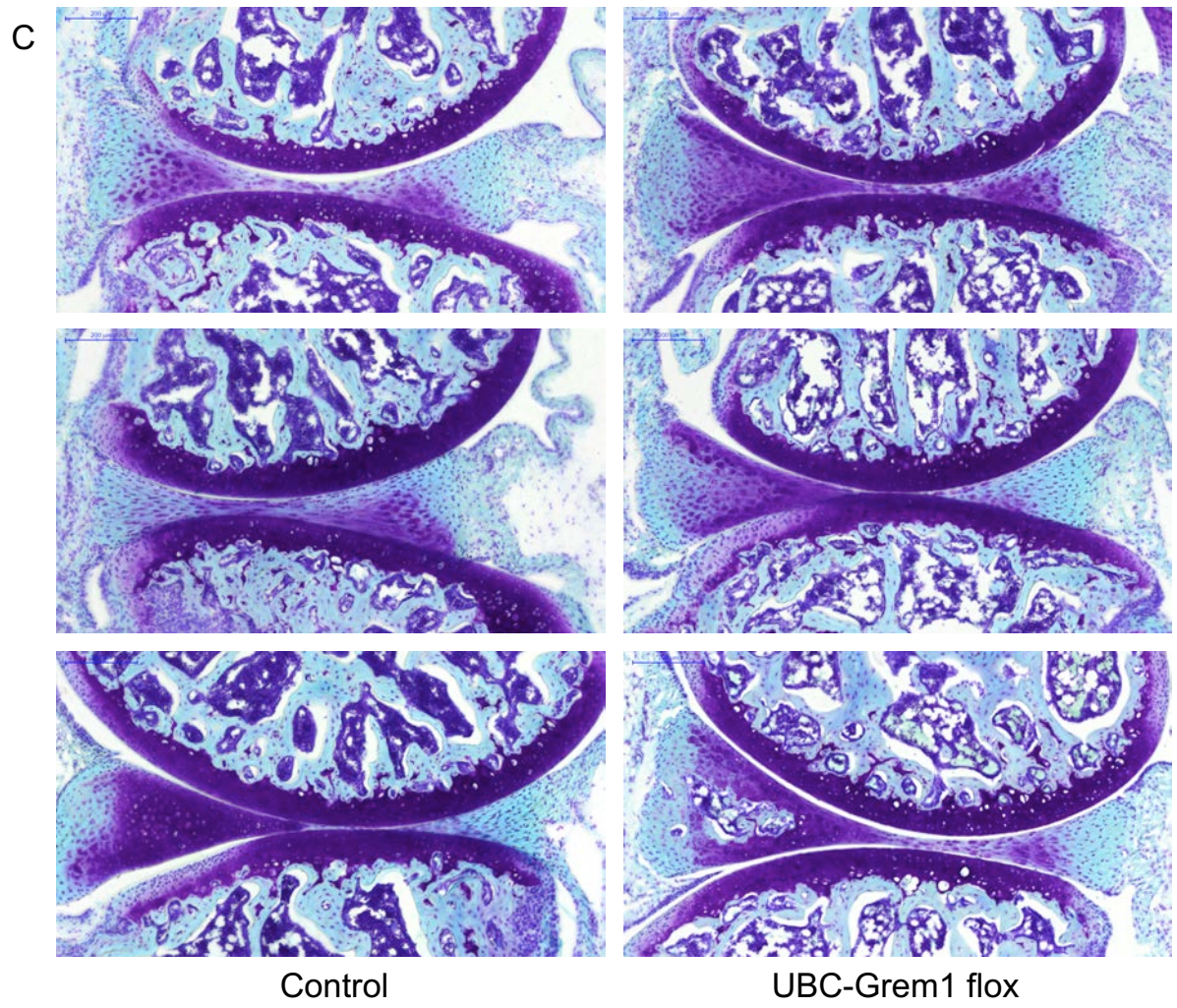
The integrity of articular cartilage was analysed by toluidine blue and fast green staining to identify the proteoglycan (purple coloured) expressed within the structure. Staining showed no observable differences in proteoglycan expression (Figure 5C) between the control animals and UBC-Grem1 flox animals indicating that articular cartilage was normal. There was also no notable impact on bone structure.

Further to that, other factors contributing to cellular biology of the articular cartilage, including cell proliferation as well as chondrocyte numbers within the different articular cartilage zones, were also measured. The impact of Grem1 expression knockdown on chondrocyte proliferation was investigated using PCNA staining and quantification was presented as total percentage of PCNA positive cells in the articular cartilage (Figure 5D) and percentage of PCNA positive cells within the individual zones (Figure 5E). The findings demonstrated that Grem1-expression has no impact on the total number of proliferating articular chondrocytes as well as proliferating chondrocytes in the different zones. Further to this, no significant differences were observed in the percentage of chondrocyte numbers within the different zones of the articular cartilage (Figure 5F).

A


UBC-Grem flox
Grem1:GAPDH expression
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
-0.0002
****
Cre Neg
Cre Pos


B
Control
UBC-Grem1 flox
20x
40x
20x
40x
Marrow
Articular cartilage
i
ii
iii
iv
v
vi
vii
viii
ix
x
xi
xii


C
Control
UBC-Grem1 flox

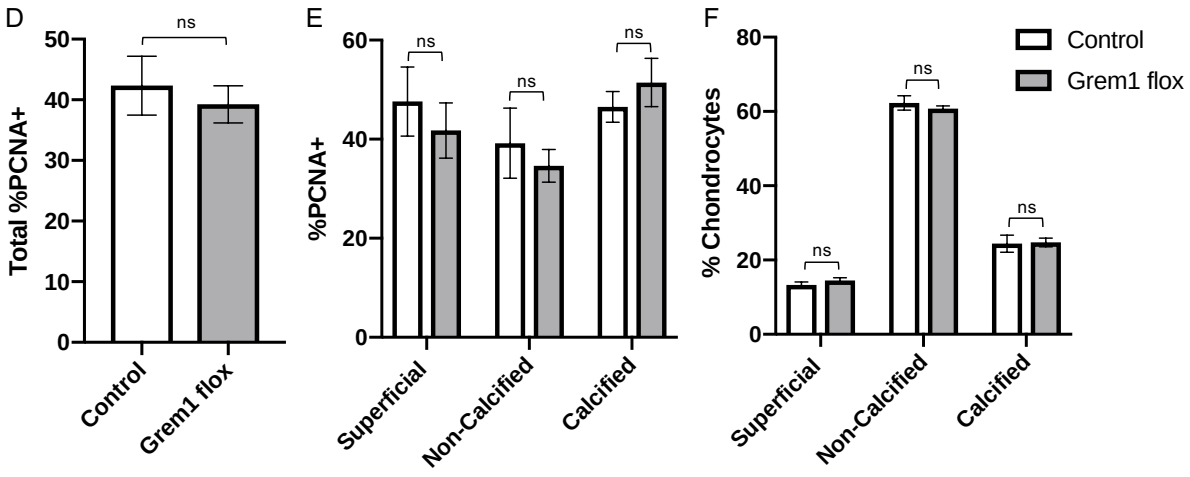

D
Total %PCNA+
50
40
30
20
10
0
ns
Control
Grem1 flox
E
%PCNA+
60
40
20
0
ns
ns
ns
Superficial
Non-Calcified
Calcified
F
% Chondrocytes
80
60
40
20
0
ns
ns
ns
Control
Grem1 flox
Superficial
Non-Calcified
Calcified

Figure 5. (A) qPCR showing a significant knockdown of Grem1 expression in the experimental animals with Cre Neg as the experimental control. (B) Representative images of ISH staining of Grem1 expression in the hind limbs of the animals. UBC-Grem1 flox (right) bone marrow (i – iv) showing knockdown in Grem1 expression compared to the Cre Neg control (left) at different magnifications. UBC-Grem1 flox (right) articular cartilage (v – xii) showing no Grem1 expression compared to control (left) samples. Red boxes showing positive staining. Scale bar = 20μm (20x) and 50μm (40x). (C) Representative images of toluidine blue and fast green staining showing proteoglycan integrity and bone morphology between the control (left) and experimental (right) groups. (D) Quantification of percentage of PCNA positive articular chondrocytes in all 3 zones. (E) Quantification of percentage of chondrocytes in individual zones. (F) Number of chondrocytes in individual zones quantified as a percentage of the total number of chondrocytes in the articular cartilage. A minimum of 4 independent samples per group were used to analyse all data.

4.6 Discussion

Grem1-expressing chondrocytes in articular cartilage have demonstrated an important role in the development and sustenance of healthy articular cartilage in adulthood. In addition, these cells have an important role in the progression of OA disease (unpublished data). Previous studies conducted have helped us understand the relationship of Greml expression to OA chondrocytes and their association with disease severity[52,59,61,63], however, little is known about the role of Grem1-expressing articular chondrocytes in both healthy and OA articular cartilage. The novelty of our study is that we determine the role of the newly found Grem1-expressing articular chondrocyte stem/progenitors rather than the role of Grem1 expression or secreted proteins. In this study, we aimed to investigate Grem1 expressing articular chondrocytes to determine their role in OA disease progression by ablating these cells to recapitulate OA pathology. We attempted several animal models of ablation, previously used to determine the functional role of distinct cell populations[65,66].

The DTA transgenic model was initially selected for this study as it appeared least technically challenging. The DTA model allows discreet Grem1-expressing cell ablation by mating the DTA mice with Grem1 cre-inducible mice. To quantitatively validate ablation, we further mated the Grem1-DTA mice with a TdTomato reporter mouse line so that all Grem1-expressing cells would not only express diphtheria toxin fragment A but also a red fluorescence tag upon tamoxifen administration. Unfortunately, even with the consistent dose of tamoxifen administered via the tamoxifen chow coupled with the long-term dosage of 12 weeks, no ablation was observed in the Grem1-TdTomato-DTA model. It was previously pointed out that cre-mediated recombination system that adopt the ROSA26 locus may not always be efficient at high levels in adult mice. The reason for the inefficiency may be the result of chromosomal structural modification and the increased inaccessibility to ROSA26 locus in certain type of cells following development[71].

Nevertheless, we continued the pursuit of ablating Grem1-expressing articular chondrocytes to validate their functional role in OA pathology using another transgenic animal model of ablation via the cell specific expression of diphtheria toxin receptor (DTR). The advantage of the DTR model is the ability to localise ablation of the specific cell population by injecting DT into a localised and restricted area. Our new model replaced the DTA expression with the DTR expression together with TdTomato fluorescence for validation of Grem1-expressing articular chondrocyte ablation. Again, with this model of ablation, animals were left on tamoxifen chow to induce maximum amount of DTR expression on Grem1-expressing specific cells before and during DT administration via intraperitoneal injection. Dosage and frequency of DT injections were modified from the previous study[66]. Our functional experiment yielded no significant ablation of Grem1-expressing articular chondrocytes. Two hypotheses were postulated:

1) We presumed that if Grem1-expressing articular chondrocytes are true stem/progenitor cells, then perhaps ablating them would trigger dedifferentiation from the neighbouring chondrocytes or an increase in Grem1-expressing articular chondrocytes to replace the ablated population.

Indeed, chondrocyte dedifferentiation has been well documented in OA disease and injury where collagen I and III are notably present in the fibrotic remodelling of cartilage during disease[72-75]. Collagen I and III are also markers of pre-chondrocytic mesenchymal cells in normal articular cartilage[76] and their expression indicates dedifferentiation of mature chondrocytes.

2) Since animals were left on tamoxifen chow during the course of the injections, there is a possibility that Grem1-expressing cells continued to undergo cre-mediated recombination.

We utilised PCNA staining to determine if there was an increase in proliferating cells. An increase in PCNA positive articular chondrocyte number was demonstrated, however this increase is independent of Grem1-expressing articular chondrocytes. As such, we speculated that the neighbouring chondrocytes may have undergone dedifferentiation to replace the ablated cells.

We also observed in the experiment was a high variability of the cre-mediated recombination in the Grem1-expressing population of cells. This variability can be attributed to the inconsistency in tamoxifen chow intake between individual animals, which has the potential to immensely affect the level of recombination. As described previously, in assessing transgenic animals, tissue specific recombination is by definition a mosaic due to the presence of two heterogenous genetic types within an individual[77]. Therefore, the concentration of tamoxifen administrated to the mice is directly proportional to the rate of recombination and number of TdTomato-positive cells. To overcome this variability, we changed our dosage protocol to a more discreet dose per animal. In addition, to addressing the variability caused by tamoxifen dosage, intra-articular injections of DT were also administered into the mouse knee capsule to try and counter the inaccessibility of DT into an avascular compartment. Through many efforts to counter all the above variables, our results persistently showed no significant ablation of the Grem1-expressing articular chondrocytes. This may be the result of a low expression of Grem1 in adult articular cartilage and all tissues in general[78]. One of the biggest pitfalls of transgenic ablation is the fact that specific levels of Cre protein accumulation are required for efficient recombination. Therefore, cre-mediated recombination also largely depends on the levels of Cre expression at target sites[77].

With multiple variables and the inefficiency of the Cre-mediated recombination model of ablation, we decided to create a new knock-in transgenic animal to ablate Grem1-expressing cells. Using the CRISPR gene editing technology to insert DTR and TdTomato expressions at the end of the coding region of Grem1 expression, we hoped to achieve a successful model of ablation to prove the functional role Grem1-expressing articular chondrocytes in the progression of OA disease. However, with the time constraints of this study we have yet to successfully develop a usable model.

Grem1-expressing articular stem/progenitor cells are identified by the expression of Gremlin 1. With the unsuccessful ablation of these cells, we next investigated if Grem1-secreted proteins hold a role in

OA progression. Utilising an inducible Grem1 expression knockout animal model, we were able to see the impact of Grem1 knockout at the time of tamoxifen administration. The role of Grem1 expression in limb bud formation in mouse embryos is very well understood with homozygous knockdown being embryonically lethal[79]. This lethality can be overcome with the use of homozygous knockdown of a C57BL/6/FVB mixed genetic background. Studies conducted with the mixed genetic background mouse found that a global knockdown in Grem1 expression postnatally exhibited skeletal abnormalities and caused osteopenia[80]. However, the implications of a mixed genetic background were not investigated. Furthermore, the impact of Grem1 expression on limb development was not induced postnatally. As such the impact could be downstream of a knockdown in Grem1 expression through embryonic development and cannot be categorised as postnatal. With our current study on Grem1 expression in limb development postnatally, we induced a knockout in Grem1 expression after the pups were born through another cre-mediated recombination system via tamoxifen administration. Time of induction was selected based on a previous lineage tracing study on the role of Grem1-expressing articular cells during development (unpublished data). Validation of the knockdown showed a significant decrease in Grem1 expression in whole digested bone via qPCR as well as in the marrow and articular cartilage tissues using ISH. However, the impact of this knockdown on the skeletal development was either minimal or not significance compared to the controls.

Even with the success in knocking down Grem1 expression in the transgenic animals, no significant impact on the articular cartilage proteoglycan secretion or bone structure was observed. In depth cellular analysis also showed no significant difference in proliferating articular chondrocytes or chondrocyte number both in the individual zones and articular cartilage as a whole. Our ISH staining, however, showed that most of the Grem1 expression in our transgenic model seems to be localised in the bone marrow rather than in the mature chondrocytes. Only a small population of the articular chondrocytes, mainly in the deeper zone appear to be positive for Grem1 expression in healthy cartilage, consistent with a previous study[52]. Nonetheless, it may be too early to draw conclusions from our experiment, as mice were sacrificed prematurely, during the young adolescence stage where skeletal development is

still in their early phase of maturation, as they begin to enter sexual maturity[81]. Although no conclusions could be drawn from our study, it is widely understood that Grem1 expression and secreted protein are involved in OA, joint injury as well as old age. By comparing the transcriptional differences between articular chondrocytes and growth plate chondrocytes, Grem1 is exclusively expressed in the articular chondrocytes in humans compared to growth chondrocytes[82]. As OA initiation occurs primarily in the articular cartilage, it is not surprising to note that Grem1 expression becomes significantly higher in OA patient samples compared to normal cartilage, with localisation exclusive to the superficial zone[52]. Furthermore, Grem1 expression was also shown to be significantly increased in cartilage injury (shortly after injury induction) and then gradually decreased as injury prolonged[60]. It was postulated that Grem1 may have a protective effect through the inhibition of Wnt and BMP signalling, which orchestrate chondrocyte hypertrophic differentiation[57]. Moreover, with OA being primarily an old age disease, the decrease in Grem1 expression found in old age joints may contribute to the increased incidence of OA development in relatively older patients[60]. Based on literature evidence and the above findings we speculate that there is an indication for the role of Grem1-expressing articular chondrocytes in OA as well as in initiation of repair in injury. With the new mouse model of Grem1-expressing articular chondrocyte ablation, we hope to find answers to the above posed questions in the near future.

4.7 Conclusion

Investigating the functional role of Grem1-expressing articular chondrocyte stem/progenitor cells has proven to be more difficult than we initially anticipated. To validate the functional importance of Grem1-expressing articular chondrocytes in the progression of OA we tried to ablate these cells using multiple transgenic mouse models to recapitulate the OA pathology. The commercially available mouse model of ablation using the loxP system did not seem to work with our inducible Grem1-creERT animal due to multiple reasons discussed in this chapter. With the creation of the new knock-in genetic model, we are hoping to be able to prove the relevance of Grem1-expressing articular stem/progenitor cells in the initiation and progression of OA in future.

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Chapter 5: Thesis general discussion and future considerations

5.1 Introduction

Osteoarthritis (OA) is the most common form of arthritis and it significantly and chronically affects patients' quality of life. It also heavily contributes to the nation's economic burden, costing between 1% and 2.5% of the gross national product[1]. OA is primarily associated with age but can also be aggravated with injury, trauma and obesity. The pathology is characterised by the gradual degeneration of the articular cartilage which progresses on to destruct the joint, including bone, synovium and ligaments[2]. Currently, there is no effective pharmaceutical therapy to reverse or delay OA progression. Despite the large amount of new information gleaned from in vitro and in vivo studies on chondrocyte cell biology, challenges to modify the complex processes of OA pathogenesis remain[3]. Primary and secondary prevention strategies are required to reduce the level of disability and attenuate the growing demand for total joint arthroplasty. Understanding the cellular biology of articular cartilage and bone development is eminent in finding a sustainable treatment for OA.

Even though OA is a degenerative disease, it is now recognised that inflammation plays a major role in its pathology[4-6]. The loss of articular cartilage (made up of chondrocytes) essential for the progression of OA. It also occurs within a compartment that is immuno-tolerant and accessible for cell delivery, making it a promising candidate for stem cell-based therapies. In fact, stem cell-based regenerative therapeutics have attracted a lot of attention and have been widely cited in clinical trials and preclinical models of OA[7,8]. Stem cells, mainly mesenchymal stem cells (MSCs), are the main source of cell populations considered for OA therapy. Most MSCs used in stem cell-based treatments for OA, originate from the bone marrow and adipose tissue[9]. These stem cells can be isolated from all postnatal tissues and are relatively easy to propagate in culture[10]. They are characterised by their fibroblastic shape, immunophenotype, as well as their trilineage differentiation and self-renewal potential[11]. Even

though MSCs were considered to be the origin of all skeletal tissues, MSC-based therapies for OA have shown a relatively modest and inconsistent ability to stimulate cartilage regeneration required for the effective treatment of OA. A recent discovery suggested that MSC may not be the true origin of all skeletal tissues despite their in vitro repertoire[12-15]. This formed the rationale to question if there are other populations of skeletal stem cell with enhanced regenerative properties specific to articular cartilage?

Recent research into skeletal stem cells and articular cartilage progenitors has identified a different population of stem cells that may be the key to sustainable articular cartilage regeneration. Progenitor cells identified in bovine and human cartilage, resident in the superficial layer of articular cartilage, have been shown to possess enhanced expansion properties without losing their chondrogenic phenotype[16,17]. These cells presented the same self-renewal properties and share some of the immunophenotypic profile of traditional MSCs. More importantly, they have the ability to migrate towards degenerated cartilage sites in late stages of OA[18]. Further studies in embryonic limb bud development showed evidence of the presence of more than one population of skeletal stem cells, however, the identification of these cells remains elusive without well-defined markers necessary for isolation and purification of specific cell populations[19,20].

Recently, in vivo lineage tracing enabled identification of a new skeletal stem cell population known as the Osteochondroreticular (OCR) stem cells[15]. These cells, marked by the expression of Gremlin 1 (Grem1), are distinct from the traditional MSCs as they lack the ability to give rise to adipose cells. In animal models, Grem1 expressing cells have the potential to give rise to bone tissue but more importantly, chondrocytes in the growth plate where endochondral ossification is thought to occur throughout adulthood[21]. The role of OCR cells is not only restricted to skeletal development but extended to fracture repair in adulthood. Translating the role of this new population of skeletal stem

cells in the articular cartilage and in OA could revolutionise the use of stem cell therapy to slow the progression of or halt OA degeneration.

The focus of this thesis involved investigating and characterising the role of OCR stem cells within the articular cartilage in postnatal development, adulthood and in old age. We then examined this stem cell population further to understand its role in OA disease and attempted to recapitulate OA by ablating the OCR stem cells in the articular cartilage. This study was guided by the hypothesis that OCR stem cells residing within the articular cartilage may be a novel population of articular stem/progenitor cells. Since these cells have exhibited a lineage commitment towards bone and cartilage, they may be involved in the progression of OA pathology. The aim of this project was to utilise in vivo lineage tracing and an animal model of OA to understand the involvement of OCR cells in articular cartilage and OA in a mission to discover novel therapeutic means for stem cell-based treatment of OA.

5.2 Discussion and Future Considerations

5.2.1 Grem1-expressing articular cartilage stem cells contributed to cellular architecture and stem cell biology of articular cartilage in development and adulthood.

Skeletogenesis is defined by the well-established process of endochondral ossification whereby epithelial and mesenchymal cells interact to form cell condensation[22]. Cell condensation is described as the fundamental cellular unit of morphological change in organogenesis during vertebrate evolution by which more than one bone and cartilage arises from a single condensation complex[23-25]. The condensations, extensively made up of chondrocytes, lay down the foundation of cartilage and matrix for osteoblasts invasion[26]. This process continues throughout adulthood, where chondrocytes undergo hypertrophy, and forms the basis of bone growth by directing the formation of mineralised matrix, angiogenesis and apoptosis[26]. Articular cartilage is established when the subchondral growth front approaches the articulating surface of the joint and stabilises, defining the thickness of the articular cartilage. The articular cartilage is crucial for the joint, serving its primary function for smooth gliding and protection of the subchondral bone from mechanical stress[27]. Due to the avascular nature of the structure, articular cartilage is notoriously known for its poor repair capacity once the cartilage has begun to degenerate. Understanding the cellular architecture and stem cell biology is pivotal to the development of stem-cell based therapy for articular cartilage disease and injury.

This thesis (Chapter 2) evaluates the role of Grem1-expressing stem cells by characterising them in the articular cartilage, both during postnatal development as well as adulthood, establishing these cells as articular chondroprogenitor or stem cells. Unlike the previously reported Gdf5[28] and Prg4[29,30] articular stem/progenitor cells that gave rise to articular cartilage structure during embryonic development, Grem1-expressing stem cells demonstrated, via lineage tracing, that they give rise to articular cartilage in postnatal development and further maintain the structural integrity in adulthood. The progenies from

these stem cells populated chondrocytes from all three zones in the articular cartilage and differentiated into multiple different cell types in postnatal skeletal maturation. More importantly, Grem1-expressing articular cartilage cells isolated specifically localised within the epiphyseal joints of adult mice long bones exhibited properties of self-renewal and multilineage differentiation in vitro. This is of particular importance as it indicates that the Grem1-expressing articular chondrocytes isolated in adulthood, albeit a heterogenous population, maintained properties of stemness and an enhanced chondrogenic differentiation capability. This may not be the first-time articular cartilage stem/progenitor cells were identified, however the therapeutic potential of the previously identified population of Gdf5 and Prg4 cells in embryonic articular structures still remains to be elucidated. Identifying markers necessary for isolation and purification of the articular cartilage stem/progenitor cells in adulthood is crucial as the first step to harnessing their therapeutic potential for articular cartilage regeneration.

Additionally, we investigated the contribution of other skeletal stem cell populations in formation of articular cartilage and bone during development as well as in adulthood. In line with previously reported data[12-15], we have shown that the traditional MSCs, marked by the expression of LepR, did not give rise to many articular cartilage chondrocytes, but substantially contributed to the formation of bone and perivascular cells within the bone marrow. This further substantiates the modest outcome of the current MSC-based therapies for OA, as traditional MSC are not the origin of articular cartilage and thus cannot adequately repair the tissue. The minimal repair reported was mostly constituted by fibrous cartilage which cannot sustain the long-term mechanical wear and tear that is required of the articular joint[31,32].

Further to this, Grem1-expressing articular stem/progenitor cells have not been found in articular cartilage in old age. This finding is particularly significant considering the positive correlation of joint degeneration disease with increased age. As such, it is reasonable to propose that loss of Grem1-expressing articular stem/progenitor cells may hold a role in initiation and increased risk of degenerative cartilage disease.

Further consideration is required to investigate the mechanism of Grem1-expressing articular cartilage stem cells in giving rise to the chondrocyte within the structure. Staining of PCNA or 5-Ethynyl-2'-deoxyuridine (EdU) dosing in the early tracing during both, the development and adulthood, would demonstrate whether these cells proliferate to self-renew and differentiate to become somatic skeletal cells. Quantification of long-term tracing of adult bones, coupled with PCNA staining, should further elucidate whether these cells differentiate and self-renew to support interstitial and appositional growth mechanism.

5.2.2 Grem1-expressing articular cartilage stem cells is lost in OA.

As pathology of OA is initiated by the loss of articular cartilage it was a natural progression of the study that we next investigated this novel population of articular cartilage stem cells under disease conditions. Damage to the joint in OA is induced by a complex interplay of genetic, metabolic, biochemical, and biomechanical factors leading to the activation of an inflammatory response and ultimate joint destruction[33]. Despite the thorough understanding of metabolic activities and inflammation mediators that lead to the progression of the disease, the lack of efficient treatment is perplexing. With the new discovery of adult articular cartilage stem cells, new therapies can be developed. Understanding their involvement in the complex cellular processes that regulate the physiological and pathological functions of the neighbouring chondrocytes in OA, is essential to the development of more effective strategies for stem cell-based OA treatment.

The next phase of our study (Chapter 3), assessed the fate of Grem1-expressing stem cells in articular cartilage in comparison to:

1) traditional MSCs marked by LepR and

2) chondrocytes marked by Acan

during the progression of OA. We aimed to understand the cellular process of early and late stages of OA by utilising a surgically induced mouse model of OA. In this disease model, the transection of the medial meniscotibial ligament was performed to dislocate the medial meniscus of the joint, leading to destabilisation of the knee thus creating a state of pathology, mimicking human OA, via natural wear and tear of joint movement. The cellular processes involved in OA showed that disease progression could be partly attributed to the loss of proliferating articular chondrocytes in all three zones in the cartilage early into the disease. The decrease in proliferating chondrocyte numbers could also be observed in both superficial and non-calcified zones of late stage OA even though it may not have been significant in comparison to that during the early stages of disease. The non-significant loss of

proliferating chondrocytes in the calcified zone in later stages of OA may be an indication of an association with an increase in hypertrophic chondrocytes, mostly constituting the calcified zones of articular cartilage. Normalised data displayed a positive correlation of an increase in hypertrophic chondrocytes in the calcified zone with progression of OA.

This chapter further addresses the involvement of individual cell populations using transgenic animals to trace the fate of skeletal stem cell subsets in the progression of OA. In addition to the loss of proliferative chondrocytes driving the progression of disease, Grem1-expressing articular stem/progenitor cells were also significantly decreased in number in late stage OA. This finding indicates that the loss of Grem1-expressing articular cells may contribute to the progression of OA. Further to this, areas abundant with of Grem1-expressing cells coincided with areas of increased proteoglycan staining. This implied that Grem1-expressing chondrocytes hold a role in proteoglycan secretion, which provides compressive resistance and shock absorption within the articular joint. Proteoglycans are essential in the protection of articular cartilage from the progressive degeneration in OA. Conversely, Acan-expressing articular chondrocytes showed no involvement in OA, presenting no significant loss in number throughout the stages of disease. As Acan marks all chondrocytes in the articular cartilage, observation of no significant loss further substantiates that progression of OA is due specifically to the loss of Grem1-expressing articular chondrocytes and not just any chondrocyte cell type. Although LepR-expressing cells did not give rise to any articular chondrocytes and, therefore, could not have had a role in the initiation of OA disease, they are, at least in part, involved in the formation of the osteophyte-like structure in later stage OA.

To allow for a better understanding of our observations, we performed single cell RNA (scRNA) sequencing analysis of cells isolated from our lineage tracing mice. scRNA analysis on Grem1-lineage articular cartilage cells showed that these cells expressed high levels of Prg4 (proteoglycan 4/ lubricin), which has been previously identified as an embryonic articular cartilage progenitor[29]. Furthermore,

DCN (decorin) which is also another proteoglycan that together with Prg4, is found primarily in extracellular matrix of articular cartilage were also expressed exclusively in the Grem1 articular chondrocyte population. LepR cells on the other hand expressed primarily genes involved in inflammatory response and myeloid cell dysfunction, as well as bone marker Col1a2 (collagen type 1 alpha 2). Our scRNA analysis further explains the poor regenerative property of traditional MSCs for articular cartilage as they do not display chondrogenic properties in adulthood.

In line with literature showing a correlation between Grem1-expression and OA severity, future experiments of scoring the different stages of OA pathology should be considered. This would enable us to draw a correlation between OA severity and the loss of Grem1-expressing articular chondrocytes. Although the link between Grem1-expression and OA severity has previously been reported[34-37], this study is the first to investigate the relationship between Grem1-expressing cells and OA pathology, rather than the role of Grem1 genetic expression in disease. More experiments are needed to look into the role of LepR-expressing cells in the osteophyte-like formation to determine the mechanisms of these cells in OA osteophyte progression.

5.2.3 To validate the role of Grem1-expressing articular stem/progenitor cells in articular cartilage health and OA progression.

In order to validate the importance of Grem1-expressing articular stem/progenitor cells in articular cartilage health and OA progression, we needed to demonstrate that the loss of Grem1-expressing cells in articular cartilage impacts the integrity of the tissue resulting in an eventual OA pathology. Animal models of a Cre-inducible diphtheria toxin (DT) mediated cell lineage ablation had been previously used to validate involvement and significance of lineage specific cells. Two animal models of diphtheria toxin, namely the diphtheria toxin fragment A (DTA) and the diphtheria toxin receptor (DTR) are commercially available for scientific use. The inducible DTA model actuates cell lineage specific ablation by the expression of DTA under the control of a loxP-flanked stop cassette in the ubiquitously expressed ROSA26 locus. Cre-mediated recombination activity, where Cre expression is induced by tamoxifen administration, removes the stop cassette leading to the expression of DTA causing cell death[38]. This model of ablation is straight forward with minimal manipulation of the animal thus is highly popular for the systemic ablation of the specific cell lineage, however, localised and site-specific ablation is impossible due to the nature of this model. On the other hand, the Cre-mediated DTR ablation model allows for site-specific ablation of a targeted cell lineage by the expression of DTR rather than the toxin itself. Meaning that diphtheria toxin (DT) injection is required for the ablation of the targeted cell lineage, where site-specific ablation is achievable and is preferred for cell lineage ablations that are lethal.

In this chapter (Chapter 4), we attempted to make use of both of the above-mentioned transgenic animal models to ablate Grem1-expressing stem cells in articular cartilage to recapitulate OA pathology. Grem1 has been shown to be poorly expressed in adult articular cartilage and all tissues in general[39]. Throughout this study, we encountered multiple challenges attempting to recapitulate OA pathology in the transgenic animals through ablation. Numerous methods and models were employed to overcome the low expression of Grem1 in adulthood. Cre-mediated lineage tracing allows for the tracking of

targeted cell population and their cell fate, thus understanding the nature and role of these cells within the complex interplay in vivo. The Cre-mediated recombination system for ablation was inadequate due to the following variables.

1) Cre-recombination that adopts the ROSA26 locus can be inconsistent in adult mice due chromosomal structural modification and the inaccessibility to ROSA26 locus in certain cell type following development[40].

2) Certain levels of Cre protein accumulation are required for efficient recombination in transgenic animals and this can be directly attributed to the tamoxifen dosage as well as the target expression level[41]. With Grem1 being poorly expressed in adulthood, it is not surprising that it has failed to accumulate the amount of Cre protein required for successful recombination and expression of DTA and DTR for ablation.

With the pitfalls of the Cre-loxP recombination system, we created another transgenic animal ablation model using the CRISPR/Cas9-gene editing technology[42]. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9- technology has recently revolutionised genetic editing in organisms where it was previously impossible to do so[43]. The technology employs the Cas9 nuclease to introduce a break in specific site of the target DNA using a guide RNA. Utilising the host's cellular DNA damage repair pathway, the mutation can then be integrated into the host genome by high-infidelity homology-directed repair mechanisms in the presence of an alternative repair template[44-46]. CRISPR/Cas9 technology facilitated generation of transgenic animals with precision and ease, and more importantly, reduced the time required to generate and breed transgenic animals via the traditional targeted embryonic cells knockout method[47]. With our newly designed transgenic animals, DTR expression alongside fluorescence TdTomato expression was localised in cells actively expressing Grem1. DTR, Grem1 and TdTomato were efficiently transcribed into a fusion protein driven by only one promoter. Post-transcriptional modification then allowed for the folding of each individual protein by cleaving at the 2A site, thus, providing equivocal amount of multiple protein expression in the same

cells and tissues[48]. The development of this animal model, however, could not be achieved due to the time constraints of this project.

Unable to investigate the functional impact of Grem1-expressing articular chondrocytes through ablation, we decided to look at the impact of Grem1 expression on the articular cartilage and bone development. Since Grem1 expression is fundamental in identifying this population of articular cartilage stem/progenitor cells, loss of expression may be what is required to change articular cartilage and bone tissue structure and integrity. Grem1 expression was successfully knocked down and validated using qPCR and in situ hybridisation within our transgenic animal model. Results showed that despite the knockdown of Grem1 expression in whole bones, the marrow and articular cartilage, there was no significant effect on bone, articular cartilage structure or extracellular matrix content. We further analysed this at the cellular level to assess any changes involving articular chondrocytes. Quantification of the total number of articular chondrocytes and the rate of proliferation remained unaffected in all three zones within the articular cartilage. Although this chapter yielded no further information to validate the importance of the cell subset in maintaining articular cartilage structure and health as well as the progression of OA, our efforts provided a level of understanding of articular cartilage cellular biology and confirmed low levels of expression of Grem1 in adulthood, particularly in the tissues of articular cartilage.

5.3 Thesis Conclusion

In summary, this project investigated cellular biology of populations within articular cartilage using transgenic animal models to map the cell fate of Grem1-expressing stem cells. Throughout the study we discovered a new population of articular cartilage stem/progenitor cell (Chapter 2) involved in skeletal modifications in early postnatal stages as well as throughout adulthood. This population of cells was lost in articular cartilage of old age, implying their importance in maintaining articular cartilage health, possibly through the regulation of proteoglycan secretion. As OA and articular cartilage disease often occurs in old age, our investigations further reinforced the idea that loss of the Grem1-expressing articular stem/progenitor cells, perhaps through the lack of chondrocyte proliferation, may accelerate articular cartilage degeneration and the progression of OA (Chapter 3). These novel findings however were hindered by the unsuccessful attempts in validating the importance of the stem/progenitor cells in propelling OA disease through ablation. We hope that with the help of a new animal model (currently in development), we would address this gap in knowledge (Chapter 4). Nevertheless, the discovery of the 'true' articular cartilage stem/progenitor cells may be the key in revolutionising stem cell-based therapy for OA, by creating efficient and sustainable regeneration of articular cartilage and thus reversing the progression of OA and cartilage disease in the future.

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