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Articular Cartilage-Derived Stem Cells: Identification, Characterisation and their Role in Spontaneous Repair

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Abstract

The poor reparative potential of articular cartilage is largely attributed to its avascular and aneural status combined with low cellularity; chondrocytes only occupy 10% of the tissue volume. Consequently, there have been a number of strategies developed to augment repair most notably through microfracture and Autologous Chondrocyte Implanation (ACI). However, both of these techniques have limitations. In the case of microfracture, the repair tissue is often fibrocartilaginous and in ACI, the number of cells that can be generated that maintain chondrogenicity is limited thus restricting the size of defect that can be treated. Consequently, there has been increasing interest in the use of Mesenchymal Stem Cells (MSCs) for the treatment of cartilage defects. Here, we discuss the isolation and characterization of a tissue-specific stem cell population from articular cartilage and the potential for its use in intrinsic and extrinsic repair of articular cartilage lesions. Significantly, unlike MSCs isolated from bone marrow, upon differentiation into the chondrogenic lineage, these cells fail to terminally differentiate i.e. are not endochondral, failing to synthesize both collagen type X and alkaline phosphatase. Senescence of chondrocytes following injury or as part of aging is hypothesized to be integral to degenerative disease and correlates with significant telomere erosion within chondrocytes. Articular cartilage-derived stem cells exhibit detectable telomerase activity and exhibit reduced erosion of telomeres; therefore, we hypothesize maintenance of this population is critical to tissue homeostasis.

Introduction

"If we consult the standard Chirurgical Writers from Hippocrates down to the present age, we shall find, that an ulcerated cartilage is universally allowed to be a very troublesome disease; that it admits of a Cure with more Difficulty than carious Bone; and that, when destroyed, it is not recovered", William Hunter [1].

Articular cartilage (AC) is both avascular and aneural. Whilst the lack of a nerve supply contributes towards pain-free locomotion, it also fails to alert of damage to the tissue upon injury. It can also be argued that the lack of a blood supply contributes towards a limited reparative response to injury. Failure to respond to such injuries may lead to secondary arthritis as a downstream consequence often requiring total or partial joint replacement. Thus, over the recent years, considerable effort has gone into devising a variety of therapies to augment the repair of cartilage. These have included cell therapies such as autologous chondrocyte implantation (ACI) in a range of guises that include a variety of scaffold types, to pure biomaterial implants designed to promote repair [2,3]. More recently, attention has turned to stem cells as a source for reparative procedures [4]. Because stem cells can be expanded extensively, it can be argued that larger lesions could be addressed principally because differentiated chondrocytes quickly lose their phenotype in monolayer culture following approximately seven population doublings, and, only a fraction of these cells re-express a chondrogenic phenotype upon redifferentiation [5]. In an attempt to circumvent this problematic *in vitro* aspect of chondrocyte behavior, some groups have utilised bone marrow-derived stromal cells (BM-MSC) as a source for cartilage repair [6,7]. However, one has to contend with the inherent problem with BM-MSC as a cell source in that they are innately endochondral that is, they have a propensity, under chondrogenic conditions, to terminally differentiate and calcify as during bone fracture healing [8,9].

Identification and Isolation of Putative Tissue-Specific Stem Cells

Through our understanding of early developmental growth mechanisms of AC we came to the conclusion that the principal vector of growth was appositional rather than interstitial [10]. This finding led us to hypothesize the existence of stem cells near or at the surface of AC that directed growth through control of cellular division and differentiation. Our initial work to clarify this hypothesis was based upon earlier studies on the development of AC using the South American opossum, *Monodelphis domestica* [11]. The reason for this choice of model was that although neonates are born with precociously developed forelimbs, the hindlimbs are more akin to those seen *in utero* in mammals such as mice. Thus, one has access to be able to manipulate and study limb and joint growth from early developmental stages that would require surgery in non-marsupial mammals [11]. Our preliminary experiments using *M. domestica* neonates involved injection of bromodeoxyuridine into the synovial knee joint of hind limbs in order to track cell proliferation in developing AC [10]. Many chondrocytes within the bulk of the cartilage tissue labeled after the first few days post-injection, but after further repeated injections we observed a small cohort of cells at the articular surface that also labeled with bromodeoxyuridine, (Figure 1). Whilst there may be a number of explanations for such a pattern of labelling, we predicted that these latter chondrocytes might be in an extended cell cycle time, a feature of stem cells, and thus, labelling in these cells only became apparent after several injections [12]. In 1999, our laboratory was the first to isolate cloneable cells from the surface layer of the 7-day bovine AC and also found that these cells were not present in the deeper layers. Colony forming cells were isolated by differential adhesion to fibronectin, a twenty minute incubation on fibronectin coated culture dishes was sufficient to isolate stem cells producing colonies in excess of 32 cells that could be easily differentiated from Transit Amplifying

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(TA) cells that produced smaller colonies of less than 33 cells, i.e. TA cells are restricted to no more than five population doublings. In 2004, we reported on the isolation and partial characterisation of a stem cell from the metacarpophalangeal joint of 7-day-old bovine AC. (Figure 2A) [13]. Similar and complimentary findings were published the same year by Martin Lotz's group who identified human tissue-specific stem cells in normal and osteoarthritic cartilage on the basis of cell surface expression of CD105 and CD166 [14]. Further work identified the Notch signaling pathway to be central in the regulation of clonality, with Notch1 prominent in expression at the surface and also in setting the boundary of the tissue, (Figure 2B-2D), [13,15,16]. We also discovered that unlike differentiated chondrocytes that lose their chondrogenic potential when expanded in monolayer culture, human AC stem cells maintain chondrogenicity after extensive expansion through continual synthesis of the transcription factor Sox9 [17,18]. Whilst in 17 and 18 we had cautiously labelled our cells as progenitors

Figure 1: Bromodeoxyuridine (BrdU) incorporation into developing articular cartilage. Mid-sagittal paraffin wax sections through the proximo-distal axis of the knee joint of *M. domestica* showing the effect of BrdU incorporation on articular cartilage growth 1 month post-injection. [**A**, **B**] Comparative fluorescence and phase contrast images [**A**, **B**, respectively] showing the distribution of BrdU in the distal femoral condylar cartilage. BrdU is incorporated into a small number of surface zone cells (*arrows*) and into a relatively greater proportion of underlying transitional zone cells.
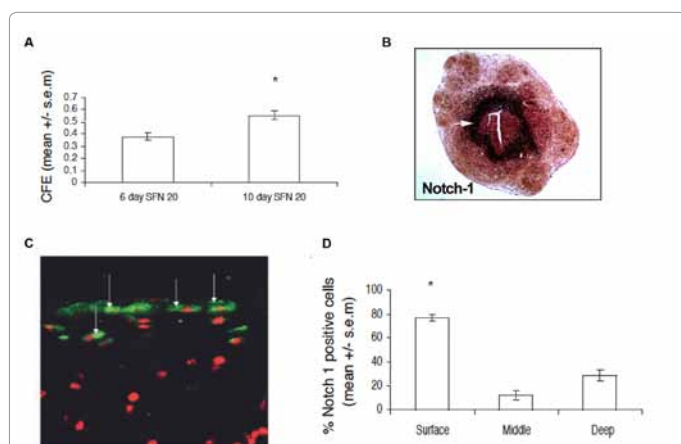
Figure 2: Isolation of articular cartilage-derived stem cells. [**A**] Surface zone chondrocytes were subjected to differential adhesion to fibronectin and colonies consisting of more than 32 cells were counted at 6 and 10 days and the results expressed as colony forming efficiency (CFE). Colonies initially appeared at 6 days in samples derived from the surface zone and the CFE of this cohort had increased by 10 days. [**B**] Chondroprogenitors cultured for 21 days in chondrogenic medium demonstrate Notch1 labelling (arrowed). [**C**] Seven-day bovine articular cartilage labelled with antibody to Notch1 (*green*) and counterstained with propidium iodide (*red*). Chondrocytes within the uppermost 2-3 cell layers of the surface zone (*arrows*) label strongly for Notch1. [**D**] Chondrocytes were labelled with anti-Notch1 antibody and subjected to single channel FACS analysis immediately after isolation; 86% of surface zone cells label positively for Notch1 compared with 10% and 34% from middle and deep zone, respectively. (*$P < 0.001$; compared with middle and deep.).

to take account of the changing definitions attributable to multipotent and pluripotent cells, subsequent work has confirmed our earlier thinking and so that these cells are in fact stem cells and fall within the minimal criteria of a stem cell or MSC [19]. Sox 9 is a key factor in regulating chondrogenesis and whose gene expression rapidly declines in culture expanded, dedifferentiated, full-depth chondrocytes [17,18]. The mechanism(s) whereby Sox9 expression is lost by chondrocytes remains unknown to date.
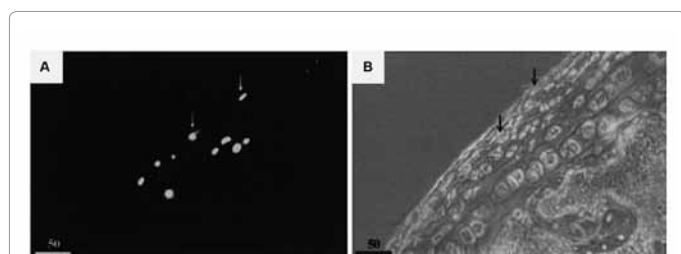
Properties of Tissue-Specific Articular Cartilage-Derived Stem Cells

Colony forming chondrocytes isolated from AC are classified as Mesenchymal Stem Cells (MSCs) since they conform to the minimal criteria for MSCs in that they are adherent to plastic, express cell surface markers CD105, CD73, and CD90, do not express haematopoetic cell surface markers such as CD34 and CD45, and can undergo multipotent differentiation to osteoblasts, adipocytes or chondrocytes [13,18-20]. In common with other MSCs, AC-derived stem cells also exhibit the property of immune-privilege, they are MHCI$^+$, MHCII$^-$, CD40$^-$, do not express T-cell co-stimulatory antigens CD80 or CD86 and are immunosuppressive when used in the mixed lymphocyte reaction assay [20]. Thus, fibronectin-positive colony forming cells isolated from AC constitute a stem cell population although it is likely that progenitor-like cells may also be present within an expanded heterogeneous population. Most critically, in terms of repair and regeneration, when AC-derived stem cells are cultured as high density pellets in chondrogenic medium they do not terminally differentiate, i.e. they maintain their phenotypic status of permanent cartilage [20]. In contrast, using the identical assay, bone marrow-derived stromal cells express transcription factor Runx2 and high levels of collagen type X and matrilin-1 [20]. Thus, AC-derived stem cells, whilst being functionally equivalent to bone marrow-derived MSCs, have a restricted differentiation potential and are unable or are limited in their capacity to undergo hypertrophy and mineralisation.

In relation to immune-privilege, several groups have reported on the use of induced pluripotent stem cells as a cell source for cartilage repair procedures derived from patient dermal fibroblasts, chondrocytes or synoviocytes from Osteoarthritis (OA) patients [21-23], the rationale being that using such sources as an autologous therapy would avoid immune rejection [24,25]. However, in a landmark paper by Xu and colleagues, it was found that genetically identical ES or iPS-derived cell lines formed teratomas and could also elicit an immune response when re-implanted into mice [26]. Thus, caution is required when pluripotent cell sources are used as a repair strategy, in the latter case, indicating the need to derive such cells to an adult fate [27].

Stem Cells and Injury

The nature of AC; its avascularity, the high electronegativity of its extracellular matrix and low cellularity preclude rapid repair of joint surface defects [28]. For intrinsic repair to occur three prerequisites have to be met; chondrocytes have to be activated - either increase their synthesis of reparative extracellular matrix components or dedifferentiate, they have to proliferate in order to provide and maintain repair tissue, and, they have to indirectly or directly migrate to the source of repair. There is evidence to suggest intrinsic mechanisms of repair do evoke a reparative response following experimental wounding of AC [reviewed in 28]. In the study conducted by Namba et al. [29] superficial wounds were made in the trochlear groove of foetal lambs *in utero*, a sequential repair process was observed, initially with the formation of a hypocellular repair tissue that was subsequently

populated with chondrocytes. If sub-chondral experimental wounding is larger than a critical diameter, >6mm, intrinsic spontaneous healing is attenuated [30]. However, if wounding breaches the subchondral bone plate of the joint (as happens deliberately during microfracture-induced repair) then infiltrating BM-MSCs initiate a relatively rapid repair response of small or large lesions that is characterized by being fibrocartilaginous and, consequently, not particularly durable in this high stress biomechanical environment [31]. There is no direct evidence to show that cartilage-derived stem cells participate in repair of chondral lesions, and is partly due to the complete lack of specific biomarkers for these cells. However, studies have noted the prevalence of Notch1 activated chondrocytes either in wounded or osteoarthritic tissue [32,33].

Following single-impact loading and in the presence of fibroblast growth factor-2 (FGF2) chondrocytes repopulating the surface of mature equine cartilage are found to be positive for Notch1 and proliferating cell nuclear antigen [32]. The same study also tentatively described 'activated' chondrocytes as being progenitor-like in character and provided histological evidence for possible migratory activity of these cells. In *ex vivo* culture of immature AC, experimental wounding of explants and culture in FGF2 causes chondrocyte cluster formation, (Figure 3A), and simultaneously induces activation of Notch1 expression within cells of the clusters, (Figure 3B-3C), [34]. If Notch signalling is activated in FGF2 treated wounded cartilage using recombinant Jagged ligand, Notch1 activated cells immediately undergo apoptosis. It is not known whether addition of soluble ligand subsequent to cluster formation is similarly deleterious to cell function. Notch1 expression would be predicted to occur in chondrocyte clusters in osteoarthritic tissue, a finding that we and others have confirmed in human tissue (Figure 3D-3F), [35]. An increase in Notch1 expression in chondrocyte clusters in osteoarthritic cartilage is also coincident with an increase in stem cell numbers [14]. The expression of Notch1 is not consistently found in all chondrocyte clusters, (Figure 3B-3C), the significance of this observation needs to be investigated in combination with the known phenotypic variability in chondrocyte aggregates [36].

The cellular density between fibrillated and non-fibrillated OA cartilage has been shown in morphometric studies to be unchanged [37], and in the absence of any histological evidence of cellular division, aberrant migration of chondrocytes to form cell clusters is hypothesised to lead to cartilage fissuring due to lack of maintenance of intervening extracellular matrix [38]. Definitive proof of chondrocyte migration *in vivo* is still lacking, however, the differential expression in OA and normal cartilage of Netrin signaling components that are implicated in cell motility and axonal guidance seems to suggest that this may be a fruitful avenue of research [39]. It is noteworthy that the cells within the clusters show both mature and immature phenotypes, some cells expressing CD105/CD166 and Notch1 whilst others may be positive for collagens type II and X [33,40,41] An alternative explanation may be that initially the individual activated chondrocytes regress to an immature stem cell-like phenotype that then generates the cluster, and which then differentiates upon loss of Notch1. It is clear that much more effort is required to resolve these conundrums for us to better understand, mechanistically, the responses to cartilage injury.

Telomere Dynamics, Stem Cells and Senescence

Given the lack of specific biomarkers for articular cartilage-derived stem cells and MSCs in general, methods of tracking populations both *in vitro* and *in vivo* are limited. As stem cells exhibit significant replicative potency, (Figure 4A), it is possible to indirectly track specific cell populations through analysis of telomere dynamics. Telomeres

are terminal regions of linear chromosomes that contain an array of tandem repeats of the DNA sequence TTAGGG that are bound by a host of telomere-specific proteins to form a specialised cap structure [42]. Telomeres function to allow chromosomal replication and prevent chromosomal fusion and rearrangements [43]. Replication of the most distal telomeric repeats is not possible, and therefore, between
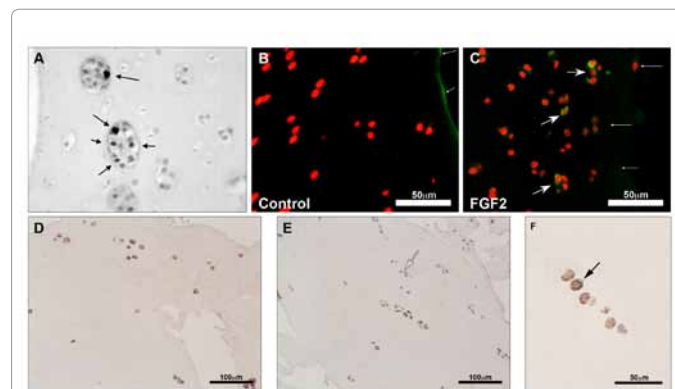
Figure 3: Notch1 expression in wounded and osteoarthritic cartilage. [A] Immunohistochemical detection of bromodeoxyuridine incorporation in chondrocytes in S phase at the wound edges of FGF treated (100ng ml⁻¹) bovine explants. Arrowheads denote the location of positively staining nuclei. Immunofluorescent detection of Notch1 in FGF2 treated bovine cartilage explant wound edges. Wounded explants cultured in the absence [B] or presence of FGF2 [C] were screened with rat anti-Notch1 (bTAN20) antibody. Anti-rat secondary antibodies conjugated with FITC (*green*) were used to reveal antibody binding. Notch1 expression was present in chondrocytes in FGF2 treated wound sites [C; thick white arrows] and either absent or weakly labelled in untreated explants [B]. Thin arrows denote the wound edge. Nuclei were counterstained with propidium iodide (*red*). [D&E] Immuno histochemical detection of Notch1 in osteoarthritic cartilage tissue sections visualized with peroxidase stain. Clusters consisted of cells that show both positive (*brown*) and negative staining for Notch1 (*arrowed*). Nuclei are counterstained blue. [F] High power image of a cell cluster in osteoarthritic tissue where all cells demonstrate Notch1 positive labelling.
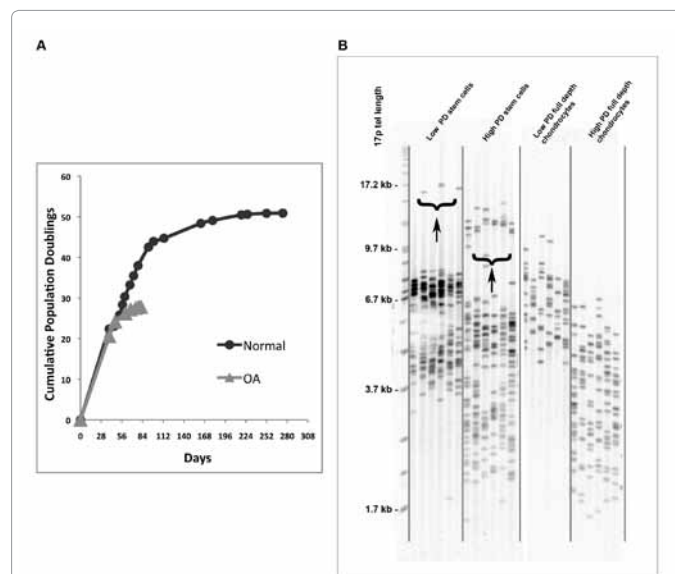
Figure 4: Characterisation of normal and OA-derived cartilage stem cells. [A] Representative growth curves of a normal and OA-derived stem cell line. [B] Telomere length assayed by Single Telomere End Length Analysis (STELA) in stem cell lines and full-depth chondrocyte populations. STELA reveals both stem cell lines and full-depth chondrocyte undergo telomere erosion [C]. It is interesting to note a subpopulation of cells in the stem cell line show a larger distribution which is increasing with time in culture (*arrowed*).
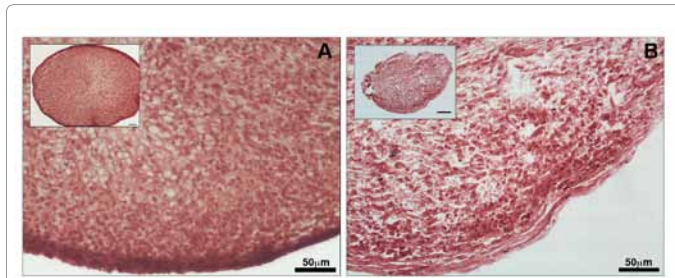
Figure 5: Chondrogenic pellet culture of normal and OA-derived stem cells. Stem cell lines were cultured in high density pellets in chondrogenic media for 28 days then processed for histology. **[A]** Stem cell pellets from normal donors exhibited extensive labelling for Safranin-O, that stains proteoglycan glycosaminoglycan chains in the extracellular matrix. **[B]** OA-derived stem cell pellets were anemic in comparison.

50-150bp are lost following each cycle of genomic replication and cellular division [44]. As telomeres shorten, cells reach their replicative limit and enter senescence, thereby preventing potential chromosomal and genomic instability that may provide the impetus to induce cellular immortalization [45]. In some cells, telomeric ends are maintained by a ribonucleoprotein complex composed of telomerase and an RNA primer sequence [46]. During embryogenesis, cellular levels of telomerase are high, but in later developmental stages, telomerase synthesis is down-regulated in most cells of the body with the exception of low-level activity in stem cells and higher activity in germ cells [47,48]. Work by Jody Buckwalter's group demonstrated that telomere shortening and the proliferative capacity of chondrocytes correlated with donor age of AC tissue, and as well as intrinsic replicative mechanisms of telomere shortening, they identified extrinsic mechanisms that also regulate erosion, principally oxidative stress [49-51]. It is hypothesized that replicative stress or 'exhaustion' following cartilage injury increases the risk of OA later in life through accumulation of senescent chondrocytes that are unable to repopulate acellular tracts of cartilage, are unresponsive to anabolic growth factors such as insulin growth factor-1 and produce extracellular matrix proteins that disrupt tissue homeostasis and therefore optimal biomechanical function [52-55].

We have observed that immature bovine-derived AC stem cells exhibit detectable telomerase activity and undergo delayed telomeric erosion during *in vitro* monolayer culture whereas dedifferentiated chondrocytes derived from a full-depth tissue isolate with significantly lower levels of telomerase activity exhibit telomeric erosion at approximately 20 population doublings [17]. Cartilage-specific stem cells from adult human donors with normal cartilage also display greater telomerase activity than full-depth chondrocyte isolates, and as these cell lines undergo further population doublings the difference in telomerase activity grows 7-fold [18]. This increase in human articular cartilage-derived stem cell telomerase activity following monolayer expansion correlates with the appearance of a subset of high molecular weight bands using Single Telomere Elongation Length Analysis (STELA), suggesting that a subset of stem cells is gradually being amplified, (Figure 4B), [18]. Osteoarthritic cartilage-derived stem cell clones have, in contrast, significantly reduced cell potency, both in terms of their replicative potential, (Figure 2A), and also in their chondrogenic potential, (Figure 5A-5B).

Many questions concerning the role of stem cells and their correlation with telomere dynamics particularly in the context of disease require answers; such as, whether stem cell dysfunction is integral to osteoarthritic disease, is stem cell dysfunction related to premature or replicative senescence, is dissolution of the stem cell niche [56]

critical in inducing replicative senescence? We are currently working to address the above questions through detailed analysis of monoclonal stem cell lines derived from patients undergoing arthroplasty.

In conclusion, the finding of an innate population of MSCs within AC, that are not overtly endochondral when differentiated into chondrocytes offers a unique opportunity for application in cartilage repair procedures. Furthermore, since these stem cells display high replicative potency, we can contemplate strategies that can address much larger lesions than is currently available using standard ACI procedures. Also, greater understanding of the role and regulation of articular cartilage-derived stem cells during growth, development and disease may allow us to intervene, facilitate and control intrinsic reparative responses.

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