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Cell origin, volume and arrangement are drivers of articular cartilage formation, morphogenesis and response to injury in mouse limbs

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ABSTRACT

Limb synovial joints are composed of distinct tissues, but it is unclear which progenitors produce those tissues and how articular cartilage acquires its functional postnatal organization characterized by chondrocyte columns, zone-specific cell volumes and anisotropic matrix. Using novel *Gdf5^{CreERT2}* (*Gdf5-CE*), *Prg4-CE* and *Dkk3-CE* mice mated to R26-*Confetti* or single-color reporters, we found that knee joint progenitors produced small non-migratory progenies and distinct local tissues over prenatal and postnatal time. Stereological imaging and quantification indicated that the columns present in juvenile-adult tibial articular cartilage consisted of non-daughter, partially overlapping lineage cells, likely reflecting cell rearrangement and stacking. Zone-specific increases in cell volume were major drivers of tissue thickening, while cell proliferation or death played minor roles. Second harmonic generation with 2-photon microscopy showed that the collagen matrix went from being isotropic and scattered at young stages to being anisotropic and aligned along the cell stacks in adults. Progenitor tracing at prenatal or juvenile stages showed that joint injury provoked a massive and rapid increase in synovial *Prg4*+ and CD44+/P75+ cells some of which filling the injury site, while neighboring chondrocytes appeared unresponsive. Our data indicate that local cell populations produce distinct joint tissues and that articular cartilage growth and zonal organization are mainly brought about by cell volume expansion and topographical cell rearrangement. Synovial *Prg4*+ lineage progenitors are exquisitely responsive to acute injury and may represent pioneers in joint tissue repair.

1. Introduction

Synovial joints are complex, diverse and anatomically fitted structures that are of obvious and crucial importance for body function and quality of life (Archer et al., 1999; Mow and Sugalski, 2001). Articular cartilage in particular has received a great deal of attention owing to its essential role in joint movement, but also its disease susceptibility and poor repair capacity (Caldwell and Wang, 2015; Huey et al., 2012; Johnstone et al., 2013). In adults, the tissue displays a zonal organization and an abundant and unique extracellular matrix (Hunziker et al., 2007). The superficial zone consists of flat tightly-bound cells that sustain frictionless joint motion by producing hyaluronan and Prg4/ lubricin (Jay et al., 2001). The intermediate zone is made of oval chondrocytes oriented randomly and separated by appreciable matrix. The deep zone is thick and made of large, round chondrocytes aligned in vertical columns perpendicular to the articular surface and separated by abundant inter-columnar resilient matrix. These structural and organizational features are needed for articular cartilage function (Hunziker et al., 2007; Mow et al., 1992), but it remains unclear how the tissue acquires and maintains them normally (Decker et al., 2014b). It is also not fully clear from where joint progenitors originate and how they bring about tissue formation, growth and morphogenesis.

In newborn mouse knees, the entire prospective articular cartilage is a thin and dense tissue consisting of 6–8 layers of small, randomly-oriented *Proteoglycan 4* (*Prg4)* and *Tenascin-C* (*TnC)* expressing cells with scant matrix (Iwamoto et al., 2007; Rhee et al., 2005). It is only over postnatal life that the tissue grows in thickness and acquires its distinct zonal organization and cell columnar arrangement (Gannon

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et al., 2015; Julkunen et al., 2009), while the total number of cell layers remains about the same. How this transition occurs is a question that has vexed researchers for years. The discovery of cells with a progenitor character in the superficial zone of adult articular cartilage was received with a great deal of interest, particularly as a possible tool to enhance tissue repair (Dowthwaite et al., 2003; Williams et al., 2010). It also led to the suggestion that similar progenitors would be present in neonatal articular tissue and form articular cartilage by a mechanism of apposition, in which the progenitors would proliferate and produce vertical columns of daughter cells encompassing the full thickness of mature cartilage (Dowthwaite et al., 2003; Hunziker et al., 2007). This model was further examined in recent cell lineage studies using knock-in (heterozygous null) *Prg4*^{GFPCreERt2} mice (Kozhemyakina et al., 2015). Following tamoxifen injection at E17.5, *Prg4* ^{GFPCreERt2}/*R26-LacZ*-positive (*Prg4*+) cells were found to form a single surface layer in incipient articular tissue at P0, become more numerous over time and then produce full-thickness columns of articular chondrocytes by 6–8 weeks of age. Though plausible, this model leaves several questions unanswered, including why only the most superficial cells would have this function, why cells would adopt different volumes in different zones over time, and how the cells would become oriented along specific axes. Given the importance of these questions, we reexamined them using new transgenic *Prg4* ^{CreERT2} (*Prg4*-CE), *Dkk3* ^{CreERT2} (*Dkk3*-CE) and *Gdf5* ^{CreERT2} (*Gdf5*-CE) mice, in addition to *Gdf5Cre* mice we originally used to identify the mesenchymal interzone as the initial birth site of embryonic limb joint progenitors (Koyama et al., 2008). Our data do not wholly sustain a model of appositional growth. Rather, we find that articular cartilage growth and thickening mainly rely on formation of non-daughter cell stacks and cell rearrangement, with limited contribution by cell proliferation and a major role played by zone-specific cell volume increases. We also find that embryonically- or adult-generated CD44+/P75+ progenitors cells with *Prg4* lineage character in synovium appear to be exquisitely sensitive to acute cartilage injury.

2. Materials and methods

2.1. Mouse strains

Commercial strains from Jackson Laboratory were: *Gt(ROSA)26Sor*$^{tm9(CAG-tdTomato)}$; *Gt(ROSA)26Sor*$^{tm6(CAG-ZsGreen1)}$; *R26R-Confetti Gt(ROSA)26Sor*$^{tm1(CAG-Brainbow2.1)Cle}$; *Prg4*^{GFPCreERt2}; *Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$; and *Gt(ROSA)26Sor*^{tm1Sor}. *Gdf5*Cre and *Prg4*mCherry mice were described previously (Decker et al., 2014a; Koyama et al., 2008). Animal experiments were approved by the IACUC at our respective Institutions. The various sets of data described in the present study were gathered and verified in a minimum of six independent experiments.

2.2. Generation of BAC transgenic Dkk3-CE, Gdf5-CE and Prg4-CE inducible Cre mice

2.2.1. DNA constructs

Murine BAC clones RP23-55N5, RP23-158D24, and RP24-400O24 containing the genes for *Prg4*, *Dkk3*, and, *Gdf5* respectively were obtained from the Children's Hospital Oakland Research Institute (CHORI). pLD53.SC2-EGFP and PSV1.RecA vectors were kindly provided by Gong et al. (2010). pCAG-CreERT2 was obtained from Addgene (gift from Dr. Connie Cepko, plasmid #14797).

2.2.2. Assembly of pLD53.SC2-CreERT2

Using pCAG-CreERT2 as a template, CreERT2 was PCR amplified using Phusion DNA polymerase (Finnzyme) and cloned into the pLD53.SC2 after double digestion with Not1 and Sac1 restriction endonucleases. During this cloning procedure, the multiple cloning site was modified to contain Not1, Swa1, BsiW1, and Mlu1 upstream of a Kozak sequence and translational start site using the oligonucleotide sequences, Forward 5′-TCACGCGGCCGCATTTAACGTACGACGCGTT GAGCCGCCACCATGTCCAATTTACTGACC-3′ and Reverse 5′-CACTGA GCTCTATCAAGC TGTGGCAGGGAAACCCTCTGCCT-3′ for PCR amplification.

2.2.3. Cloning of homology arms into pLD53.SC2-CreERT2

Homology arms for *Prg4*, *Dkk3*, and *Gdf5* were synthesized by high-fidelity PCR, gel purified and restriction digested. For *Prg4*, a 625 bp region of homology one nucleotide upstream of the translational start site was amplified from RP23-55N5 using oligonucleotide sequences Forward 5′-CTCTGGGCCCGCTATATAAGACTTCCAGCACA CTGGAGA-3 and Reverse 5′-CTCTACGCGTGTTCTCGGATGCAACGCC CTTGCTTGAGA-3′ containing PspOMI and Mlu1 restriction endonuclease sites. For *Dkk3,* a 564 bp region of homology immediately upstream of the translational start site was amplified from RP23-158D24 using oligonucleotide sequences Forward 5′-CTCTGCGGC CGCATCGCGATGGGAGGAGGATG-3′ and Reverse 5′-CTCTACGCG TGTTTGTTCCGCGCTGGCCGCCGCCTGTG-3′ containing Not1 and Mlu1 restriction endonuclease sites. For *Gdf5,* a 215 bp region of homology one nucleotide upstream of the translational start site was amplified from RP24-400O24 using oligonucleotide sequences Forward 5′-CTCTGGCGCGCCGTCTGGACACGGGAGCACTTCCACT-3′ and Reverse 5′-CTCTACGCGTCTCTGGCCAGCCGCTGAATGACACC AC-3′ containing Asc1 and Mlu1 restriction endonuclease sites. Using standard cloning methods, homology arms for *Prg4* and *Dkk3* were cloned into pLD53.SC2-CreERT2 after its digestion with Not1 and Mlu1, while the homology arm for *GDF5* was cloned into the Mlu1 and Asc1 sites of pLD53.SC2-CreERT2.

2.2.4. Bacterial recombination to introduce CreERT2 into desired BAC clones

Recombinase A was introduced into host bacteria containing RP23-55N5, RP23-158D24, and RP24-400O24 by transformation with pSV1.RecA vector (100 ng) and selected for on chloramphenicol (12.5 μg/ml)+tetracycline (10 μg/ml) LB agar plates. Bacteria containing RecA were then transformed by electroporation with 1 μg (1–2 μl) of the pLD53.SC2-CreERT2 containing the appropriate homology arm for the specific BAC clone. SOC medium (1 ml) was added and transformed bacteria were incubated with shaking at 200 rpm for one hour at 30 °C. Recombinants were first selected for by adding 5 ml of LB medium containing chloramphenicol (12.5 μg/ml), ampicillin (50 μg/ml), and tetracycline (10 μg/ml) to bacteria and grown overnight at 30 °C with shaking at 200 rpm. Further selection for recombinant clones was carried out by plating 100 μl of overnight culture onto chloramphenicol (12.5 μg/ml)+ampicillin (50 μg/ml) LB agar plates and incubated overnight at 42 °C. Chloramphenicol and ampicillin resistant colonies were screened by colony PCR using primers flanking the 5′ end of the homology arm: recomPrg4 Forward 5′-GG ACTAATTGGTTCATCCCAGTCCA-3′, recomDkk3 Forward 5′-GG TGGTCATCGTCGTGGAGATAG-3′ and recomGdf5 Forward 5′-TTTCAGCTGCTGACTGGAGACG-3′ and all combined with the primer recomCreERT2 Reverse 5′-CTGGCCCAAATGTTGCTGGATAGTTT TTACT-3′. Colony PCR identified candidate recombinants, which were further verified by diagnostic restriction enzyme digestion and field inversion gel electrophoresis.

2.2.5. BAC purification and pronuclear injection

BAC constructs were purified from 100-ml cultures using a Maxi Kit (Qiagen) with minor modifications. During alkaline lysis, 2 M potassium acetate was used in place of a 3 M solution. Additionally, after elution with QF buffer, two phenol/chloroform extractions followed by one chloroform extraction were carried out to further clean up the BAC DNA. BAC DNA (12 μg) was then linearized with Not1-HF restriction endonuclease (NEB) for 4–5 h and loaded onto a CL-4B Sepharose (Sigma) column pre-equilibrated with injection buffer (10 mM Tris,

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