CRISPR/Cas9 knockout of HAS2 in rat chondrosarcoma chondrocytes demonstrates the requirement of hyaluronan for aggrecan retention

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Abstract

Hyaluronan (HA) plays an essential role in cartilage where it functions to retain aggrecan. Previous studies have suggested that aggrecan is anchored indirectly to the plasma membrane of chondrocytes via its binding to cell-associated HA. However, reagents used to test these observations such as hyaluronidase and HA oligosaccharides are short term and may have side activities that complicate interpretation. Using the CRISPR/Cas9 gene editing approach, a model system was developed by generating HA-deficient chondrocyte cell lines. HA synthase-2 (*Has2*)-specific single guide RNA was introduced into two different variant lines of rat chondrosarcoma chondrocytes; knockout clones were isolated and characterized. Two other members of the HA synthase gene family were expressed at very low relative copy number but showed no compensatory response in the *Has2* knockouts. Wild type chondrocytes of both variants exhibited large pericellular matrices or coats extending from the plasma membrane. Addition of purified aggrecan monomer expanded the size of these coats as the proteoglycan became retained within the pericellular matrix. *Has2* knockout chondrocytes lost all capacity to assemble a particle-excluding pericellular matrix and more importantly, no matrices formed around the knockout cells following the addition of purified aggrecan. When grown as pellet cultures so as to generate a bioengineered neocartilage tissue, the *Has2* knockout chondrocytes assumed a tightly-compacted morphology as compared to the wild type cells. When knockout chondrocytes were transduced with Adeno-ZsGreen1-myc*Has2*, the cell-associated pericellular matrices were restored including the capacity to bind and incorporate additional exogenous aggrecan into the matrix. These results suggest that HA is essential for aggrecan retention and maintaining cell separation during tissue formation.

Introduction

One event associated with osteoarthritis (OA) is the loss of aggrecan proteoglycan from the extracellular matrix (ECM) of articular cartilage [1–3]. In addition to aggrecan, a significant loss of hyaluronan (HA) is also observed in human and animal models of OA [4–8]. For example, cultured explants of human articular cartilage treated with IL-1α displayed a loss of HA coincident with a loss of aggrecan within the same layers of the tissue [8]. Primary chondrocytes treated with IL-1β or IL-1α as models of OA, adopt an enhanced catabolic metabolism that generates a loss of the prominent HA/aggrecan-rich glycocalyx or pericellular coat [8,9]. As the loss of ECM components is associated with the enhanced catabolism of OA chondrocytes, we hypothesize that the re-establishment of a HA/aggrecan-rich pericellular matrix will provide positive signals and promote a steady-state metabolism. We further propose that HA is the limiting factor necessary for this pericellular matrix repair to be successful. To test these hypotheses we have developed a variety of approaches to selectively enhance the accumulation of cell-associated HA as well as aggrecan. What is lacking in our studies, and the field in general, is a suitable, stable, HA-deficient chondrocyte cell line—a model chondrocyte that is

also amenable to plasmid transfection or viral transduction. Experiments using primary murine, bovine or human OA chondrocytes, made HA-free by treatment with a hyaluronidase are always a race against time (6–12 h) before endogenous biosynthesis re-establishes pericellular HA levels.

We and others have used the rat chondrosarcoma (RCS) cell line as a chondrocyte model system [9–14]. RCS cells are a continuous long-term cultured line derived from the Swarm rat chondrosarcoma tumor [15,16]. Several different RCS cell lines are in use by investigators [9,12–14] and each differs in various aspects of the chondrocyte phenotype. To our knowledge, these cells lines were all derived (grown out) from tumors propagated from the Swarm rat chondrosarcoma but by different investigators at different times. The cells of the RCS cell line used often in our laboratory (labeled for this study as RCS-o) are more elongated than round and synthesize less aggrecan. However, these cells display HA/aggrecan-dependent pericellular matrices that are retained at the cell surface via CD44; retention of the HA/aggrecan-rich matrix can be blocked with the use of anti-CD44 antibodies, HA oligosaccharides [11] or overexpression of the CD44 intracellular domain [9]. These cells also exhibit the capacity for CD44-mediated endocytosis of HA [10,12]. Both of these properties are similar to what we observe using primary cultures of bovine articular chondrocytes [11,17–19]. The clustered regularly interspersed short palindromic repeat (CRISPR) technology using RNA-guided Cas9 endonuclease is a powerful gene editing tool. In 2014, Yang et al. [13] developed a Cas9 stable transfectant clone of a different RCS cell line and used these cells to generate a CRISPR/Cas9 mediated knockout of aggrecan. We obtained the wild type parental RCS cells (labeled in this study as RCS-Cas9). These cells are more round in morphology, exhibit high aggrecan biosynthesis, prominent HA/aggrecan pericellular matrices but express less CD44. Both RCS cell lines display relevant phenotypic aspects that we observe in primary cultures of articular chondrocytes and thus serve as useful models.

HA in eukaryotic cells is synthesized by one or more of three HA synthases, HAS1, HAS2 and HAS3 [20,21]. HA is synthesized directly at the inner surface of the plasma membrane and extruded through the plasma membrane into the pericellular space. HA can remain anchored to the plasma membrane by continued interaction with the HA synthase and/or via binding to cell surface receptors such as CD44 [22,23]. We have used displacement of pericellular coats or ^{3}H-labeled HA by HA oligosaccharides or unlabeled high molecular mass HA, to differentiate displaceable HA (receptor-bound) from non-displaceable HA (assumed to be HAS-bound) [11,22,24,25]. For example, during the initial biosynthesis of HA by chick embryo chondrocytes, all membrane-associated ^{3}H-HA was non-displaceable for the first 4 h, switching to predominately displaceable at times thereafter [24]. Although all three HA synthases have the capacity to synthesize HA, we demonstrated that primary human chondrocytes utilize the HAS2 synthase as the predominant HA synthesizing isoform [26]. *Has2* knockout in mice results in embryonic lethality due to disruption of cardiac development [27]. Conditional inactivation of *Has2* of early limb bud mesenchyme by introduction of the *Prx1-Cre* transgene results in skeletal deformities and severely shorten limbs due to abnormal and disorganized growth plates and a decrease in aggrecan deposition into the ECM [28]. *Has1* and *Has3* did not appear to compensate for the HA deficiency in the conditional inactivation mice although this was not determined directly.

In this study we have developed a single guide RNA (sgRNA) to target a Cas9 dependent cleavage within exon 2 of the rat *Has2* gene. We have successfully generated *Has2* mutations in two different RCS cell lines, RCS-o and RCS-Cas9—mutations that blocked the synthesis of HA in the resultant cloned cells. *Has2* knockout cells lost the ability to assemble a HA/aggrecan-rich pericellular matrix and lost the capacity to retain exogenously added, purified aggrecan. Other questions addressed were the effect of HA loss on cell–cell spacing during neocartilage formation, changes in aggrecan synthesis and retention, and the potential for compensation by the *Has1* and *Has3* synthases.

Results

Selection and screening for Has2 knockout clones

Following transfection of RCS-o and RCS-Cas9 cells with the PX458 plasmid containing a 20 nt sgRNA sequence targeting *Has2*, Cas9 and GFP, the GFP+ cells were selected and propagated following dilution cloning by the FACS cell sorter (Fig. 1, panels A and B, top left). Following growth of the individual clones, cells were analyzed for cell surface-associated HA by staining with a HA binding protein (HABP) and fluorescent microscopy. The micrographs in Fig. 1A and B, show the presence of HA by Alexa-Fluor-488 fluorescent detection of HABP on wild type RCS-o and RCS-Cas9 cells. Shown at higher magnification is the HABP staining for the RCS-o WT cells counterstained with DAPI (panel A) and the RCS-Cas9 cells that co-express mCherry (Panel B). Some of the GFP+ cells generated clones still exhibited HABP staining of cell surface HA (RCS-o *Has2* KO clones 1 and 3) and likely represent unsuccessful knockouts. However, ~80% of the GFP+ cells no longer exhibited HABP staining of cell surface HA and several were selected