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scAAV-Mediated Gene Transfer of Interleukin 1-Receptor Antagonist to Synovium and Articular Cartilage in Large Mammalian Joints

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Abstract

With the long-term goal of developing a gene-based treatment for osteoarthritis (OA), we performed studies to evaluate the equine joint as a model for AAV-mediated gene transfer to large, weight-bearing human joints. A self-complementary AAV2 vector containing the coding regions for human interleukin-1 receptor antagonist (hIL-1Ra) or green fluorescent protein (GFP) was packaged in AAV capsid serotypes 1, 2, 5, 8 and 9. Following infection of human and equine synovial fibroblasts in culture, we found that both were only receptive to transduction with AAV1, 2 and 5. For these serotypes, however, transgene expression from the equine cells was consistently at least 10-fold higher. Analyses of AAV surface receptor molecules and intracellular trafficking of vector genomes implicate enhanced viral uptake by the equine cells. Following delivery of 1×10^{11} vector genomes of serotypes 2, 5 and 8 into the forelimb joints of the horse, all three enabled hIL-1Ra expression at biologically relevant levels and effectively transduced the same cell types, primarily synovial fibroblasts and, to a lesser degree, chondrocytes in articular cartilage. These results provide optimism that AAV vectors can be effectively adapted for gene delivery to large human joints affected by OA.

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Conflict of Interest

Dr. Ghivizzani has an equity interest in Molecular Orthopaedics Inc., a company pursuing gene-based treatments for arthritic disease.

Keywords

Osteoarthritis; Self-complementary Adeno-Associated Virus; Interleukin-1 Receptor Antagonist; Synovium; Cartilage; Equine

Introduction

Osteoarthritis (OA) is a common, debilitating joint condition marked by progressive deterioration of the articular cartilage, subchondral bone sclerosis, osteophyte formation and inflammation of the soft tissues. Over time, the chronic degenerative process often leads to total joint failure and loss of mobility, necessitating prosthetic replacement. Despite the high prevalence and impact of OA, its treatment remains inadequate due in large part to a limited understanding of the pathogenesis and the biological mediators that drive the disease processes. Over the last several years, increasing evidence has emerged of an important role for interleukin-1 (IL-1) as an intra-articular mediator of cartilage loss, joint pain and inflammation in OA. In this regard, a naturally-occurring inhibitor of IL-1 signaling, the IL-1 receptor antagonist (IL-1Ra),[1] holds promise as a biologic drug for treatment. The recombinant form of IL-1Ra (anakinra) has been shown to be a useful anti-inflammatory agent in certain clinical applications,[2] however, its efficacy in treating arthritic disease has been hindered by an inability to achieve and maintain therapeutic concentrations intra-articularly.[3]

To overcome problems with effective administration of recombinant IL-1Ra, we have worked to develop technologies to deliver the IL-1Ra cDNA to cells in the synovium and capsular tissues of joints. These tissues are genetically modified to become endogenous sites of sustained, elevated IL-1Ra production, which following secretion from the modified cells, diffuses throughout the articular tissues. This gene-based approach to protein-drug delivery removes the need for repeated application while providing the greatest concentration of the therapeutic agent specifically at the site of disease.[4] We, and several others, have shown that direct intra-articular injection of certain recombinant viral vectors can provide expression of therapeutic transgenes at levels sufficient to halt experimental arthritis in the joints of laboratory animals.[5-10] Further, we have shown that with the use of immunologically compatible vectors and cDNAs, exogenous transgenes can be expressed in the joint tissues for at least 6 months.[11]

In considering vector systems to move into clinical studies for the treatment of OA, adeno-associated virus (AAV) offers several advantages over other well-characterized systems.[12] AAV is non-pathogenic; transduced cells have a low immunogenic profile, and it enables persistent transgene expression in many applications.[13] In early experiments, we found that conventional single-strand AAV vectors provided only marginal levels of transgene expression intra-articularly. More recently, we showed that self-complementary (sc; double-stranded) AAV vectors[13-15] could provide >20-fold enhancement of gene expression with rapid onset in synovial and capsular cells *in vitro* and *in vivo*.[16] We found that scAAV transgene expression levels in the knees of rabbits were similar to those provided by adenovirus, and were sufficient to mediate therapeutic responses in these animals.

Additional advances in recombinant AAV technology, including cross-packaging with alternate capsid serotypes[13] and improved methods for vector production,[17] have overcome previous technical limitations such that AAV can now be realistically considered as a candidate for human application in OA.

In humans, OA most frequently develops in the knees and hips, large joints that support the weight of the body while standing and during locomotion. Although we have shown that scAAV.IL-1Ra can effectively block experimental arthritis in the joints of small laboratory animals, the successful scale-up from rodents to humans is often a difficult challenge for gene-based treatments. *In vivo*, patterns of cellular transduction and ensuing transgenic expression are a function of the biophysical interaction of the vector with the specific target tissues. Critical, but unpredictable variables include: the volume, surface area and composition of the target tissues and extracellular matrices, the different resident cell populations and their densities, the vascularity of the tissues, the volume and composition of extracellular fluids, among others. In this respect, the small joints of a 100-200 g rodent cannot duplicate the complex milieu of the knee of a 75,000 g human. Indeed, the greater joint size and internal volume, together with the dramatically larger and thicker connective tissues, as well as the increased fluid and compressive forces generated during human locomotion can have a profound influence on the biodistribution of the injected virus, the cell populations that are effectively transduced, and the downstream, temporal patterns of transgene expression.

In an effort to model scAAV-mediated gene delivery in large human joints and, in turn, generate a more accurate depiction of its potential as a vector for use in the treatment of OA, we investigated its capacity for gene delivery to the forelimb joints of horses. The equine carpal and metacarpophalangeal (MCP) joints (located in anatomical positions analogous to the human knee and ankle, respectively) are similar in size, architecture and tissue composition to the human knee. Likewise, since these joints carry 60-65% of the horse's weight during locomotion, they are highly vulnerable to the onset of OA as a consequence of excessive loading imposed by athletic training and racing. Moreover, current diagnostic modalities and clinical treatment for OA are the same in humans and horses.[18, 19] Also, the large joints of horses permit the aspiration of milliliter volumes of undiluted synovial fluid enabling the direct measurement of therapeutic protein levels. Thus, patterns of therapeutic transgene expression in the treated joint can be directly monitored over time within the same animal.[7]

In this study, we packaged the scAAV2 vector genome in several common capsid serotypes and compared their efficiency of transduction in equine and human synovial fibroblasts in culture. Following these *in vitro* studies we selected certain serotypes for analysis of gene transfer *in vivo* following their delivery into the equine joint. The levels and duration of therapeutic transgene expression were compared, as well as the tissues and cell types transduced intra-articularly.

Results

Using scAAV vectors packaged in a battery of widely studied AAV capsid serotypes, we first compared the receptiveness to transduction of early passage synovial fibroblasts isolated from equine and human joint tissues. To enable quantitation of transgene expression we employed the coding sequences for green fluorescent protein (GFP) and human IL-1Ra (hIL-1Ra). GFP was used to determine the percentage of cells effectively transduced with each serotype, and hIL-1Ra as a secreted, quantifiable reporter of therapeutic protein expression. Cells from each species were seeded in parallel into 12-well plates, and 24 hours later were infected with scAAV.GFP or hIL-1Ra packaged in serotypes 1, 2, 5, 8 or 9 at doses in 10-fold increments ranging from 10^2 to 10^4 viral genomes (vg) per cell. The conditioned media from cultures infected with scAAV.hIL-1Ra were collected at days 3, 5, 7, and 10 for analysis by ELISA (Figure 1). Cultures infected with scAAV.GFP were analyzed for fluorescence daily by inverted microscopy, and at day 5, a subset of the cultures was examined using flow cytometry (Figure 2).

Typical of the hIL-1Ra profile shown in Figure 1 for AAV1, we found the effective vector serotypes provided rapid onset of transgene expression in both human and equine cells. Peak levels of hIL-1Ra expression were achieved by days 3-5 post-infection and were maintained through the remainder of the experiment. In both the equine and human cell cultures, serotypes 1, 2 and 5 showed the greatest production of hIL-1Ra and GFP (Figures 1b and 2), with serotype 2 providing the highest level of expression. Transgene expression from vectors packaged in serotypes 8 and 9, however, was significantly lower, at near background levels, even at the 10^4 dose.

Notably, for serotypes 1, 2, and 5, the levels of hIL-1Ra produced by the equine cells were dramatically higher (approximately 25, 9 and 50-fold, respectively) than the human cells infected in parallel (Figure 1b). Analysis of GFP expression supported these data, and showed that for each of these serotypes about 3-5 times more equine cells were effectively transduced, and expressed GFP at much greater levels, resulting in increases in mean levels of fluorescence between 18-20 fold higher than the human cells (Figure 2b).

To gain insight into the biological basis for the disparity in transgene expression between the cells of the two species, we first compared each cell type for relative expression of AAV cell surface receptor molecules. Due to the availability of antibodies with cross-reactivity between equine and human proteins we examined the cells for expression of receptor molecules for AAV2: heparan sulfate proteoglycan (the primary binding receptor) and integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (co-receptors involved in virus internalization). In parallel, equal numbers of cells from the equine and human synovial fibroblast cultures were trypsinized and incubated with each antibody individually, followed by a fluorescently tagged secondary. The cells were then analyzed for fluorescence by flow cytometry. As shown in Figure 3, consistent with their heightened transduction with the AAV2 vector, HSPG expression was found to be considerably higher in the equine cells, which appeared uniformly positive for HSPG content. HSPG expression on the surface of the human cells was not only reduced relative to the equine cells, but the absolute levels were strikingly low, just above background. Both cell types expressed high levels of $\alpha_v\beta_3$ integrin, and although

$\alpha_v\beta_5$ expression was lower than $\alpha_v\beta_3$ in the equine cells, it was not detectable over the isotype controls in the human cells.

In previous work with synovial fibroblasts from rabbits, we found that the translocation of vector genomes to the nucleus was inefficient, such that following infection the vast majority of the viral DNA within the cells remained in the cytoplasm and less than 20% had translocated to nucleus.[16] To determine if intracellular trafficking may also contribute to the differences in transduction, we performed similar vector genome trafficking studies in the human and equine cultures. As before, parallel cultures of human and equine cells were infected with ~10^4 viral genomes of scAAV.GFP packaged in serotype 5. Twenty-four hours post-infection the cells were harvested, gently lysed, and the cytoplasmic and nuclear compartments were fractionated. Viral genomes in the respective cell fractions were then quantitated using qPCR. As shown in Figure 4, approximately greater than 20-fold more viral genomes were detected in the equine cells than in the human cells; however, the relative ratios of nuclear to cytoplasmic viral DNA were the same. These data indicate that the efficiency of nuclear importation in both cell types is similarly low (<25%) and together with the surface receptor data in Figure 3, indicate that the increased transgene expression observed in the equine cells is due to increased uptake of virions, likely attributable, at least in part, to increased levels of AAV surface receptor molecules.

Having characterized several AAV serotypes for their capacity to transduce equine articular fibroblasts in culture, we selected three of these (AAV2, 5 and 8) for analysis *in vivo* following intra-articular injection into equine joints. Serotypes 2 and 5 were chosen because they provided the most robust transgene expression in culture, and several reports have examined their utility for intra-articular gene delivery in small animal models. Although serotype 8 generated only trace levels of hIL-1Ra expression in culture in either cell type, it was included as a means to test the validity of the *in vitro* assays.

Approximately 2×10^{11} viral genomes of scAAV.hIL-1Ra of each serotype were injected into both front midcarpal and metacarpophalangeal joints of different groups of horses. Since the synovial volumes of these joints (midcarpal, 14.9 ± 0.6 mL and metacarpophalangeal, 12.5 ± 1.0 mL) are nearly equivalent [7, 20], this delivery strategy enabled us to increase our sample size per animal from 2 to 4. Immediately prior to vector delivery and at periodic intervals thereafter, synovial fluid was aspirated directly from each of the joints. Recovered joint fluids were analyzed for human IL-1Ra content by ELISA, as well for leukocytic infiltration. Consistent with the *in vitro* results, scAAV.hIL-1Ra packaged in serotypes 2 and 5 generated meaningful levels of transgenic expression and produced about 1 and 1.5 ng/mL of human IL-1Ra, respectively during the first three weeks (Figure 5). hIL-1Ra expression began to diminish by week 5 and returned to baseline by week 10. Somewhat surprisingly, in contrast to our *in vitro* findings, serotype 8 also produced measurable levels of human IL-1Ra intra-articularly, at levels comparable to serotype 2, and with a similar temporal pattern.

To visualize the relative number and distribution of the cells genetically modified by the different vector serotypes intra-articularly, ~1×10^{12} vg of scAAV.GFP of each serotype were injected into the midcarpal or metacarpophalangeal joints of one horse. At day 10 post-

injection, the animal was euthanized and the capsular tissues and cartilage shavings were harvested for analysis. Consistent with the results achieved with hIL-1Ra, no meaningful differences were seen among serotypes in the phenotype, number, or locations of the cells transduced. Typical of that shown in Figure 6 for serotype 2, direct fluorescence microscopy of freshly harvested synovial tissues revealed large numbers of GFP+ cells across the entire expanse of the joint lining. The density of the fluorescent cells was greatest in the thick villous synovium immediately adjacent to the articulating cartilage surfaces (panels a and b). No fluorescent cells were seen on the opposing external surfaces of the joint capsule. Indeed, subsequent histologic section and immunohistochemical staining showed that the transduced cells were almost exclusively localized within the synovium and immediate subsynovium. GFP+ cells were rarely observed in the deeper supporting tissues.

Interestingly, and with particular relevance to the treatment of OA, direct examination of articular cartilage shavings using inverted fluorescence microscopy, showed scattered faint populations of transduced cells across the surface of about half the samples recovered from each joint (Figure 6c). Following histologic processing, analysis of these tissues in cross section showed that the transduced cells were localized primarily within the superficial zone. In some sections, however, transduced populations could be seen deeper, within the transitional zone.

Discussion

With the long term goal of developing an effective gene based treatment for OA, we performed studies to evaluate the equine joint as a model for AAV-mediated gene transfer to large, weight-bearing human joints, which are frequent sites of disease onset. *In vitro*, we found that equine and human articular fibroblasts were amenable to transduction with vectors packaged in the same capsid serotypes and were similarly resistant to others. We saw high, dose-dependent transgene expression with serotypes 1, 2 and 5, with negligible transduction from serotypes 8 and 9, regardless of vector dose. In general, our results regarding the relative transduction efficiencies of the serotypes tested are consistent with those reported previously for human and horse, individually.[21, 22] The modest differences are likely attributable to variations in methods for measuring viral titer as well as experimental conditions, including cell source, passage number and culture and infection conditions.

An intriguing finding of the present studies is the enhanced transduction efficiency of the equine synovial cells relative to those of human origin. During the process of infection, AAV vectors initially attach to the surface of target cells via specific cell-surface glycans: serotypes 1 and 5 bind sialic acid variants, while AAV2 uses HSPG. After binding, interaction with specific protein co-receptor molecules is thought to stabilize binding and facilitate virus internalization via endocytosis in clatherin pits. Once inside the cell the virus is transported in endosomes to the nucleus for uncoating and genome release. That synovial cells from both species are receptive and resistant to transduction with the same serotypes in culture, indicates that they express similar types of surface antigens, and is consistent with their common tissue origin and function. With respect to AAV2 receptors, our data show that equine synovial fibroblasts express moderate to high levels of HSPG and co-receptor

molecules, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. HSPG and $\alpha_v\beta_5$ expression on the surface of the human cells was noticeably reduced relative to the equine cells, and was only marginally detectable over background. This is somewhat surprising since, among the variants tested, the human cells were most receptive to vectors packaged in AAV2. Our genome tracking studies using AAV5 showed that following infection with the same viral dose, the equine cells contained >10-fold more vector genomes intracellularly. For both cell types though, only ~25% of the viral genomes were found in the nucleus, while ~75% remained cytoplasmic. Since intracellular trafficking and nuclear import appear similarly inefficient in the cells both species, the differences in transduction appear to occur at the level of viral entry. While we have not addressed efficiency of viral endocytosis, our data thus far suggest that increased transduction in the equine cells is due, at least in part, to heightened expression of primary binding receptor and co-receptor molecules. These phenotypic differences may be attributable to differences in the innate biology of human and equine synovial cells, or as discussed below, may possibly reflect artifact arising from adaptation to growth in monolayer.

In agreement with previous reports in mice, AAV serotypes 2 and 5 were capable of mediating effective intra-articular gene transfer in the equine joint. Following injection of 2×10^{11} viral genomes, scAAV vectors packaged in both serotypes elevated the steady state levels of human IL-1Ra in the synovial fluids to ~1 ng/mL, a level shown to have beneficial effects in other model systems.[6] Serotype 8, which generated only trace levels of hIL-1Ra expression in equine synovial cells in culture (500-1000 fold less than AAV2 or 5), was surprisingly effective and enabled transgene expression at levels comparable to these serotypes *in vivo*. Interestingly, despite the inability of AAV8 to transduce synovial fibroblasts in culture relative to AAV2 and 5, the locations and apparent cell types transduced by all three vectors intraarticularly were indistinguishable.

At present we have no definitive explanation for this result, but it is likely attributable to alterations in the expression of surface receptor molecules in synovial fibroblasts adapted to growth in monolayer relative to those in the natural context of the joint lining. For example, a known receptor for AAV8, the 67 kd laminin receptor (67LR), binds with high affinity to laminin 1 found in the basement membranes of many tissues, promoting cell adhesion and interaction of laminin with integrins.[23, 24] Expression of 67LR, however, is noted to vary significantly with growth conditions in culture.[25] Thus, while *in vitro* assays can be useful experimental tools, they are not necessarily reflective of the biology of cells in their native context. The data shown here emphasize that caution must be applied when using these methods to screen AAV serotypes for their potential as vectors for gene delivery to articular tissues. In this respect, the use of fresh tissue explants may provide a more representative depiction of the receptiveness of tissues and cell types to transduction with specific AAV capsid variants.

In the equine joint, we saw a gradual drop-off in hIL-1Ra transgene expression over a 7 week time period. Similar to previous work in rats[11], we attribute this to the xenogenic human transgene we employed as a quantifiable reporter of therapeutic protein expression. In assessing different vectors for efficiency of intra-articular gene delivery, as well as the receptiveness of different animal systems, we have frequently used this cDNA as a reference

standard for comparison. The reagents used to measure hIL-1Ra are species specific and provide unambiguous detection of the product against the background of endogenous proteins in animal models. The limitation of this transgene is that it stimulates a T-cell mediated immune response to the transduced cells, which leads to their elimination in immune competent animals, and thus, abbreviated transgene expression. Since completing the work described here, reagents for sensitive detection of the equine IL-1Ra analogue have become commercially available, and we have generated scAAV vectors containing the equine cDNA. We are currently in the process of evaluating expression of this transgene in the equine joint.

Our data show that scAAV vectors packaged in serotypes 2, 5 and 8 are capable of mediating sustained expression of exogenous transgenes at biologically relevant levels following delivery into the forelimb joints of horses. Since the equine joint is of similar size and architecture to the human knee, these early results provide optimism that similar methods can be effectively applied to achieve sustained delivery of therapeutic gene products in large human joints frequently affected by OA. The enhanced receptiveness of the equine fibroblasts to AAV transduction *in vitro* relative to their human counterparts, though, suggests that it may be necessary to administer greater doses of virus to achieve equivalent levels of transgene expression clinically. In this respect, at a dose of 1×10^{12} viral genomes, we saw no adverse response to treatment, either at delivery or any time thereafter, which suggests that considerably greater doses of vector could be safely delivered if necessary. Alternatively, it may be possible to engineer the AAV capsid to generate novel variants that target alternate surface receptors more highly expressed on human synovial cells.

The purpose for moving away from rats to studies in the equine joint is to more closely simulate gene transfer in the setting of large human joints. In this respect comparison of the transduced cell populations within the equine joint with those observed in the rat, provides interesting differences that may have important functional implications. In earlier work we saw significant movement of gene transfer vectors beyond the synovium into the ligaments and tendons of the joint capsule and peri-articular muscle.[11] The cells transduced in these nonsynovial tissues appeared to provide more stable transgene expression than those in the synovium. In the larger equine joint, with its dramatically thicker tissues, we saw little apparent movement of the vector beyond the synovial lining. It will be interesting to see how this impacts the stability of expression from a homologous, equine transgene.

An additional consideration relative to the rat, is the large volume of viscous synovial fluid in the equine joint (5-10 μls vs >10 mLs) and its potential to affect vector dispersion and ensuing transduction patterns. Moreover, as a result of natural prior infection from wild type AAV, synovial fluid in humans has been found to contain elevated titers of neutralizing antibodies to several capsid serotypes.[26, 27] The horse is not known to be a natural host for AAV, and in preliminary screens we saw no evidence of neutralizing antibody in synovial fluids of the horses prior to injection. A recent study by Ishihara et al., however, showed the capacity of intraarticular injection of AAV to stimulate a potent humoral immune response.[28] The capacity with which AAV specific antibodies in synovial fluid may neutralize a bolus of 10^{12}-10^{13} vector particles, or the ability to bypass this humoral

immunity via methods, such as by fluid aspiration or joint lavage remains unknown. The equine joint however offers a useful system in which to explore these types of practical studies, which may be of significant clinical relevance.

Our data also provide unambiguous demonstration of AAV-mediated gene delivery to articular cartilage chondrocytes *in situ*, in a joint of human proportion. Although the extent of gene transfer was somewhat modest, and variable among the cartilage samples recovered within each joint, the result is encouraging nonetheless. The therapeutic strategy explored here is based upon efficient gene delivery to the highly cellular synovial tissue, whereby cDNAs coding secreted therapeutic products are expressed and then released into the joint fluids to diffuse into the cartilage and other articular tissues. Meaningful levels of gene transfer to the chondrocytes in cartilage would likely enhance the efficacy of treatment significantly, enabling the synthesis of therapeutics directly within the diseased tissue that the treatment is intended to protect.

Materials and Methods

Construction and generation of AAV vectors

The cDNAs encoding GFP and human IL-1Ra were directionally inserted into the Sac II, Not I sites of the pHpa-trs-SK plasmid, which is a self-complementary variant derived from the genome of AAV2.[14, 15] For all scAAV vector constructs, transcription was driven by the CMV promoter/enhancer.

scAAV vectors were propagated using an adenovirus-free, two plasmid transfection system. Using 10 layer, cell factories (Nunc, Rochester, NY, USA), the respective AAV vector plasmids were co-transfected into 293 cells by $CaPO_4$ precipitation with the pXYZ packaging/helper plasmid containing the appropriate *rep* and *cap* genes.[29] Sixty hours post-transfection, cells were harvested with PBS containing 10mM EDTA, pelleted, resuspended in low salt buffer and lysed by three rounds of freeze-thaw. Cellular nucleic acids were digested by incubation with Benzonase (Sigma-Aldrich, St. Louis, MO, USA). Purification of AAV from the crude lysate was performed using iodixanol gradients followed by FPLC affinity chromatography. The eluate was desalted and concentrated with a Millipore Biomax 100K filter (Millipore, Billerica, MA, USA), aliquotted and stored at -80°C. Viral titers were determined by quantitative competitive PCR assay relative to well-characterized AAV viral reference standards. Each viral preparation was examined for purity by resolution of the viral proteins by SDS PAGE and silver stain.

Isolation and infection of primary articular fibroblasts

Equine—All procedures involving animals were conducted according to protocols approved by the University of Florida Institutional Animal Care and Use Committee. Capsular tissues from the carpal joints were harvested from an animal euthanized for health issues unrelated to musculoskeletal conditions. The non-collagenous soft tissues including the synovial lining and subsynovium, were scraped from the thick supporting tissue of the capsule using a scalpel. Under aseptic conditions, the synovium was minced with a razor blade and digested in saline solution with 0.2% collagenase for 2hr at 37°C with constant

stirring. The suspension was then passed through a nylon mesh to remove undigested tissue. The cells in the filtrate were pelleted, washed in saline and plated in DMEM supplemented with 10% FBS with 1% penicillin/streptomycin. Twenty-four hours later, the cultures were washed to remove non-adherent cells and debris; the medium was replaced, and the cultures returned to the incubator.

Human—All use of human tissues was conducted in compliance with protocols approved by the Institutional Review Board at the University of Florida. Similar to methods described above, synovial fibroblasts were harvested from capsular tissues discarded during joint replacement surgery.

For viral infection, the cells of both species were plated in 12 well plates and grown to ~70% confluence. Prior to infection, the cells were washed with serum free media (DMEM). AAV vectors from stock solutions were mixed with minimal volumes of serum free media to produce working solutions containing the desired DNAse resistant viral genomes/per cell, which were then on the cell cultures. For experiments involving GFP expression as well as intracellular trafficking, viral doses of 10^4 viral genomes per cell were used. For those involving hIL-1Ra, viral doses ranged from 10^2-10^4 viral genomes per cell. Parallel cultures of uninfected cells of each species were used as negative controls. After incubation with virus for 2 hrs, complete media was added to each well, and the cells were returned to the incubator. For quantitation of hIL-1Ra, 24 hr prior to each collection time point, the media from each well were removed, and replaced with fresh. Media harvested the following day were stored frozen at –80°C. Each viral dose was added to four individual wells, and the conditioned medium from each well was analyzed by ELISA (R&D Systems, Minneapolis, MN, USA). For quantitation of GFP expression, at day 5 post-infection, the cells were trypsinized, washed, counted and suspended in saline solution. The cells were then analyzed for levels of fluorescence by flow cytometry. Gating was set at the fluorescence intensity exceeding 95% of the population of uninfected control cells of the respective species.

Flow Cytometry

For analysis of receptor expression, adherent equine or human cells were trypsinized and resuspended in PBS/0.05% bovine serum albumin at 5×10^6 cells/mL. Cells were incubated with the specified primary antibody or isotype IgG controls; anti-integrin alpha-5-beta-3 (MAB1976H, Millipore), anti-integrin alpha-5-beta-five (MAB1961H, Millipore) or anti-HSPG (H1890-09, US Biological, Swampscott, MA, USA), at 1:20 dilution for 30 minutes at RT. Cells were then washed with PBS and stained with the appropriate secondary antibody conjugated with phycoerythrin for 30 min at RT. Finally, cells were again washed with PBS and resuspended in 0.5mL PBS prior to analysis using a LSRII flow cytometer (BD BioSciences, San Jose, CA, USA).

Quantitation of viral genomes in cytoplasmic and nuclear cell fractions

Viral DNA in cellular compartments was isolated using a procedure adapted from Zhao et al.[30] Briefly, synovial fibroblasts of each species were seeded at 10^5 cells per well in a 6-well dish, allowed to attach, and then infected with scAAV at 10^4 viral genomes/cell as described above. After 24 hours, the cells were trypsinized, incubated in hypotonic buffer

for 5 minutes on ice, and lysed in non-ionic detergent. Centrifugation of the lysate allowed the nuclear fraction to be collected as the pellet, while the supernatant was reserved as the cytoplasmic fraction. Low molecular weight DNA from each fraction was isolated by Hirt extraction[31] and then used for quantitative PCR. Primer pairs were designed to anneal to sequences within the CMV promoter sequence. Viral genomes were detected using SYBR Green dye in an Eppendorf Mastercycler Realplex2. The results were standardized to a dilution series of vector plasmid DNA of known copy number. Three independent experiments were performed, yielding similar results. Values were then expressed as the mean of these experiments.

Intra-articular gene delivery

For intra-articular delivery of scAAV, approximately 2×10^{11} viral genomes of each vector were diluted into 5 mLs of lactated ringers solution. Following surgical scrub of the forelimb joints, vector was injected directly into the midcarpal and metacarpophalangeal joints. Negative control joints received injection of lactated ringers alone. For quantitation of secreted transgene products, synovial fluid was collected from the joints by arthrocentesis at days 7, 21, 35 and 70 post-injection, and the recovered fluids were analyzed for hIL-1Ra content by ELISA. Animals receiving vectors with GFP were euthanized at 10 days post-injection, and the articular tissues were harvested and analyzed, either by directly using inverted fluorescence microscopy, or were paraffin embedded, sectioned and stained for GFP expression using immunohistochemistry.

GFP immunohistochemistry

GFP immunostaining was performed on 5μm sections of formalin-fixed, decalcified, paraffin-embedded blocks that were cut and mounted on plus charged slides (Fisher Scientific, Pittsburgh, PA, USA). Slides were deparaffinized and rehydrated through a series of xylenes and graded alcohols. Heat mediated antigen retrieval was performed using trypsin (Invitrogen, Grand Island, NY, USA) in PBS for 7 minutes at 37°C. Nonspecific binding was blocked in 10% normal serum matched to the secondary antibody species. Slides were incubated 1 hour at room temperature with a commercially available antibody: rabbit anti-GFP at a 1:200 dilution (Abcam, Cambridge, MA, USA). The appropriate biotinylated secondary antibody (Invitrogen) was applied to samples for 30 minutes at room temperature at a dilution of 1:500, slides were mounted with DAPI (Vector Laboratories, Burlingame, CA, USA), and fluorescence was visualized.

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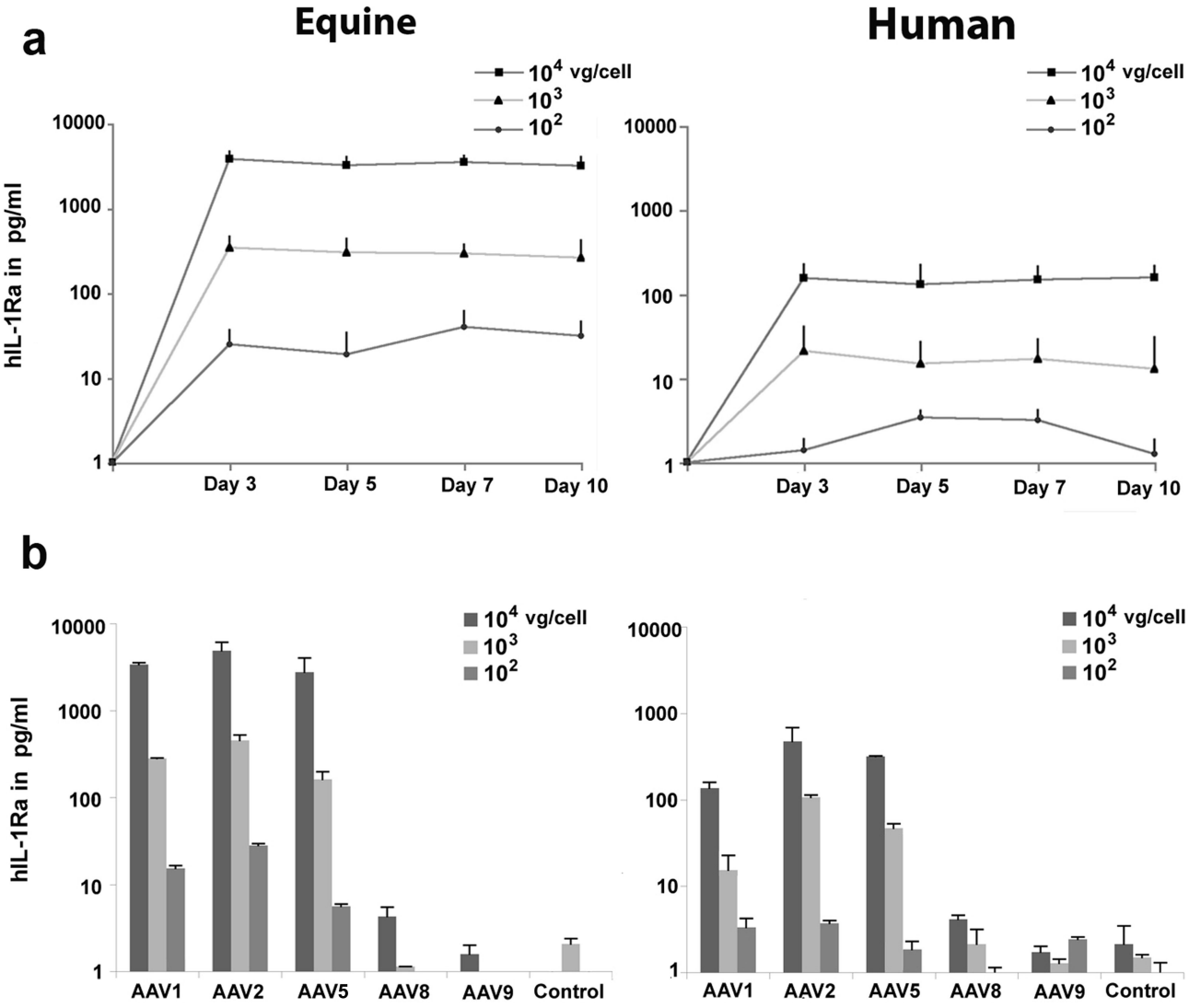

Figure 1.

Transgene expression *in vitro* following infection of equine and human synovial fibroblasts with scAAV.hIL-1Ra. Fibroblasts isolated from equine or human synovial tissue were cultured in monolayer in 12-well plates and infected with scAAV.hIL-1Ra packaged in serotypes 1, 2, 5, 8 and 9 at doses of 10^2, 10^3, and 10^4 viral genomes (vg) per cell. Conditioned media were collected post-infection at days 3, 5, 7 and 10 and analyzed for hIL-1Ra content by ELISA. (**a**) Temporal expression patterns of hIL-1Ra in both equine and human cells for AAV serotype 1. Similar patterns were observed for serotypes 2 and 5 (not shown). (**b**) hIL-1Ra production from equine and human cells at day 5 is shown for all serotypes. Values represent the mean values of 3 wells for each cell and seroptype. Error bars represent ±SEM.

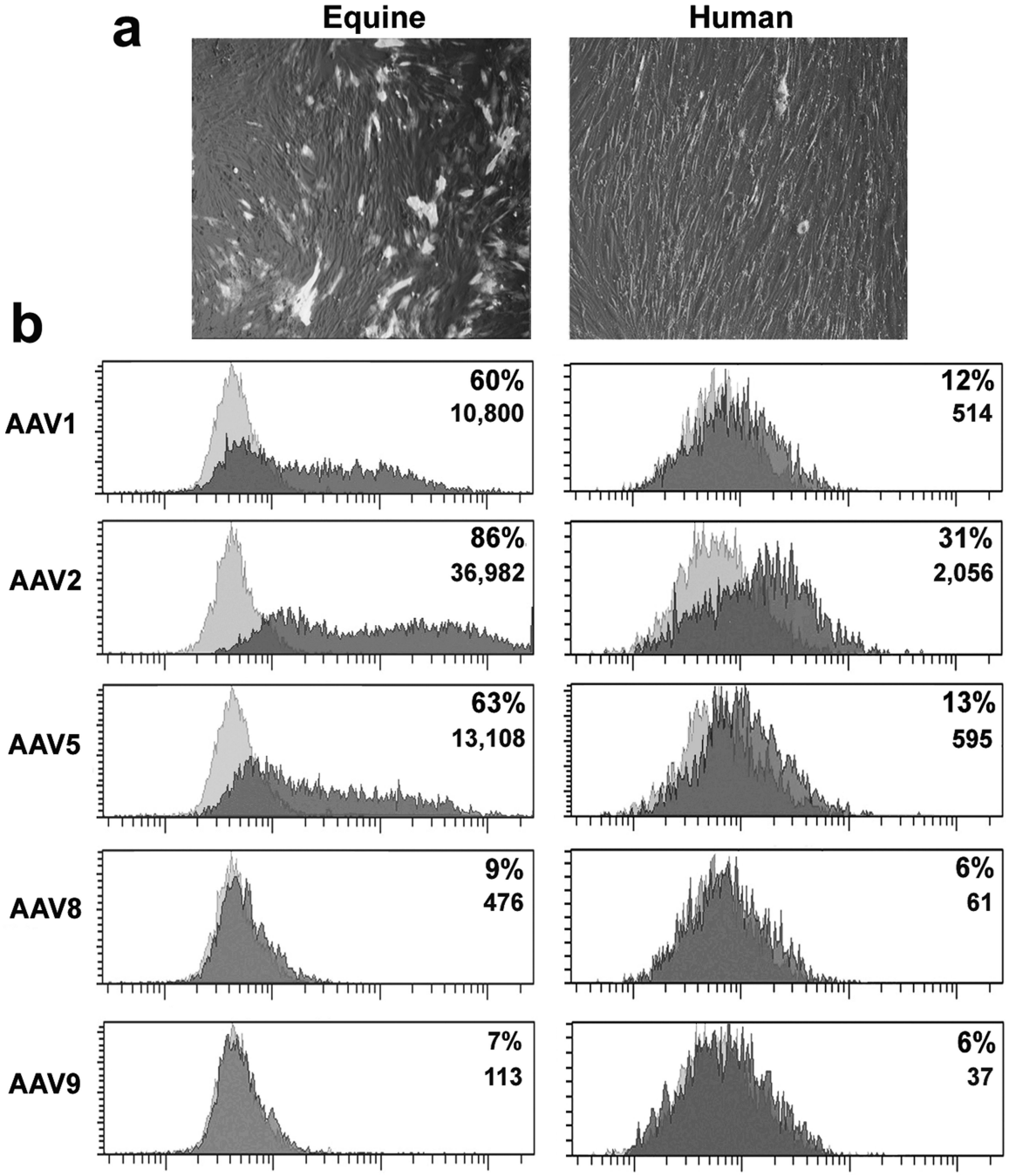

Figure 2.

Relative transduction efficiency *in vitro* of AAV serotypes in equine and human synovial fibroblasts. Fibroblasts isolated from equine or human synovial tissue were cultured in monolayer in 12-well plates and infected with 10^2, 10^3 and 10^4 vg of scAAV.GFP packaged in serotypes 1, 2, 5, 8 and 9. Images shown are from the 10^4 vg. (**a**) Fluorescence microscopy of equine and human cells at day 5 post-infection with AAV serotype 1 shows significantly greater numbers of GFP+ cells in the equine cultures. (**b**) At day 5 post-infection cultures from both species were analyzed for fluorescence by flow cytometry.

Shown are representative plots of fluorescence (horizontal axes) vs cell number (vertical axes) for each serotype. Overlays show fluorescence profiles of transduced cells (pink) relative to uninfected control cultures (gray). Percentages of the cells in the transduced cultures that exceed 95% of the cells in the uninfected controls are shown in the upper right. Increases in mean fluorescence levels over controls are shown underneath.

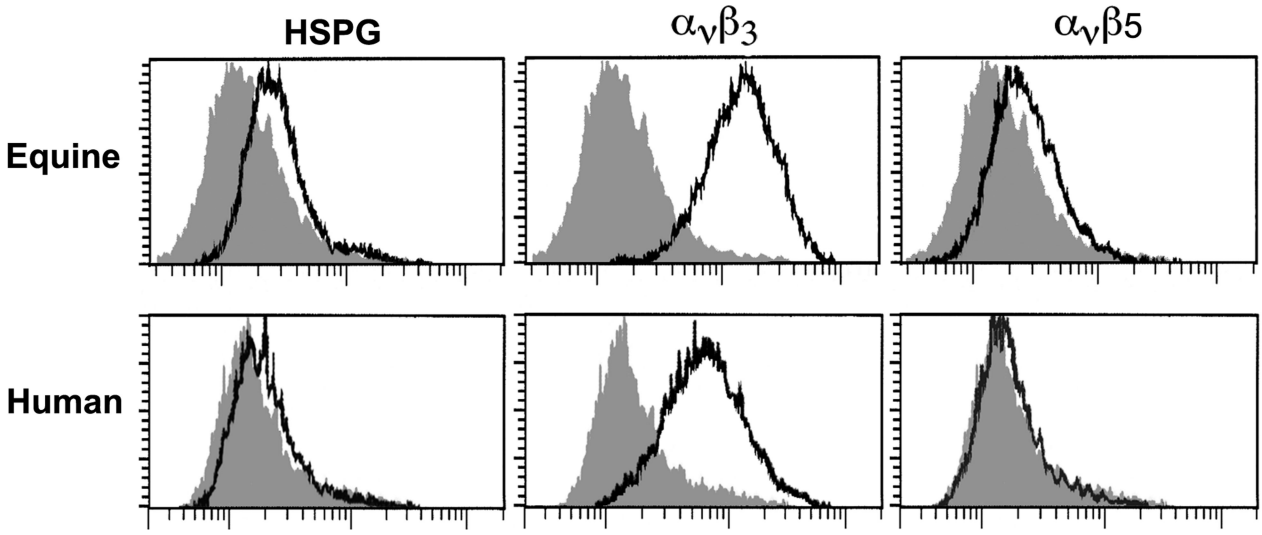

Figure 3.

Analysis of AAV2 cell surface attachment and internalization proteins. Equine and human synovial fibroblast cells were analyzed by flow cytometry for the expression of HSPG using the HepSS-1 antibody and expression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins using phycoerythrin labeled antibodies (bold lines). The grey shaded histogram (left) in each panel represents staining with isotype-matched (IgG1) control antibody.

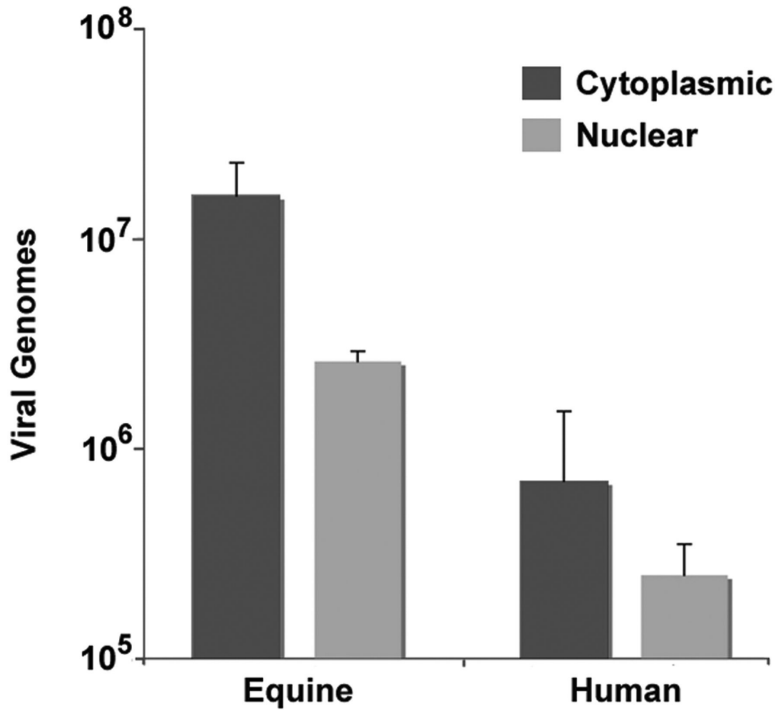

Figure 4.

Comparison of intracellular migration of scAAV vector genomes in equine and human synovial fibroblasts in culture. Parallel cultures of human and equine cells were infected with 10^4 vg of scAAV.GFP packaged in serotype 5. Twenty-four hours later, the cells were harvested, and the nuclear and cytoplasmic fractions were isolated. Viral genomes in the respective fractions were determined using quantitative PCR. Values plotted for each vector and compartment represent the means of four replicates. Error bars represent ± S.E.M. Greater than 20-fold more viral genomes were detected in the equine cells than in human. However, in both cases less than 25% of the genomes within the cell translocated to the nucleus.

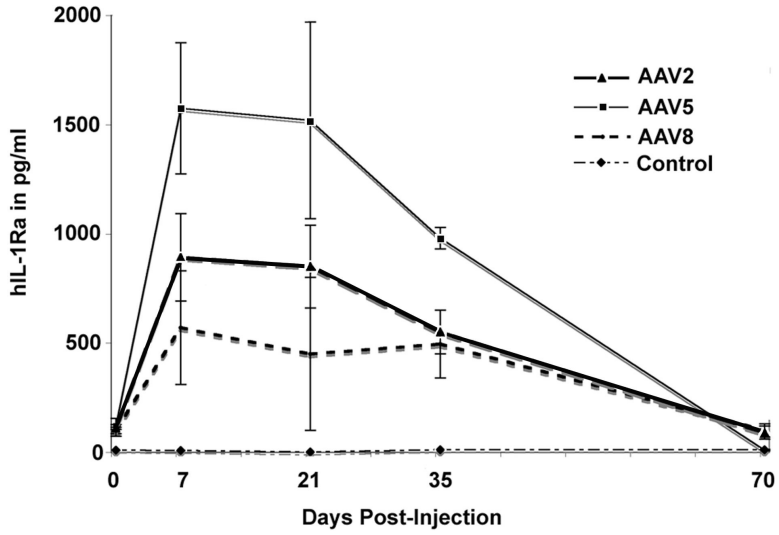

Figure 5.

hIL-1Ra levels in synovial fluid following intra-articular injection of scAAV vectors into the forelimb joints of horses. Approximately 2×10^{11} vg of scAAV.hIL-1Ra packaged in serotypes 2, 5 or 8 were injected in a random fashion into the midcarpal and metacarpophalangeal joints of both forelimbs of thoroughbred horses. One of the four joints was injected with saline and served as a negative control. At 1, 3, 5, and 10 weeks post-injection, synovial fluid was collected from the joints via arthrocentesis, and the hIL-1Ra content was measured by ELISA. Data points reflect mean values of three injected joints, with error bars indicating ± S.E.M.

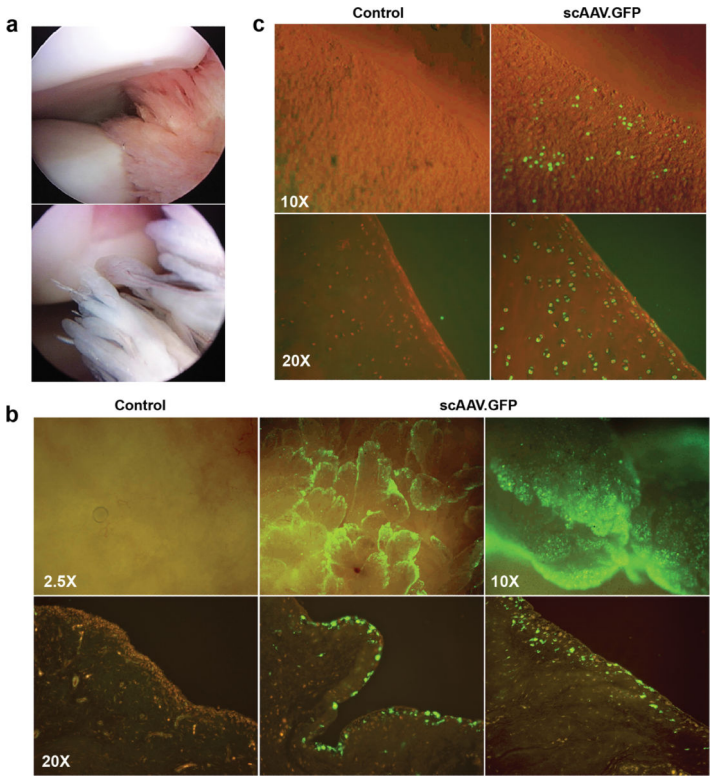

Figure 6.

Locations and phenotypes of the cells transduced by scAAV vectors following intraarticular injection in the equine joint. Approximately 1×10^{12} vg of scAAV.GFP packaged in serotypes 2, 5 or 8 were injected into the midcarpal or metacarpophalangeal joints of one horse. Following sacrifice at day 10, joint tissues were harvested and analyzed for GFP expression, either directly using inverted fluorescence microscopy, or following paraffin section and immunohistochemical staining. The images shown are from a joint injected with serotype 2, but are representative of all three serotypes. (**a**) Arthroscopic images of the interior of a healthy equine midcarpal joint are shown to illustrate the anatomy and morphology of the articular tissues. The top image shows the smooth, rounded surfaces of articular cartilage with adjacent tissues of the synovial lining. The lower image illustrates the highly villous nature of the synovium. (**b**) scAAV.GFP expression in the synovium. (**c**) scAAV.GFP expression in articular cartilage. *For both panels **b** and **c***, the images in the top row show the distribution of GFP expression across the surfaces of the freshly harvested tissues using direct inverted fluorescence microscopy. The lower panels show GFP expression in each tissue in cross section following paraffin section and immunohistochemical staining.