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ORIGINAL ARTICLE

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Recombinant adeno-associated virus preferentially transduces human, compared to mouse, synovium: implications for arthritis therapy

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Abstract Despite a number of published reports, including from our own laboratory, suggesting that adeno-associated virus (AAV) transduces mouse synovium, a careful analysis demonstrated transduction predominantly of the subsynovial muscle tissue, while the synovial lining is poorly transduced. To investigate the potential of AAV to transduce human synovium, three human rheumatoid arthritis (RA) and two murine collagen-induced arthritis (CIA) synovial cell lines were infected with recombinant AAV (rAAV) vectors encoding either mouse IL-10 or IL-4. Low-level transgene expression was observed. However, either γ-irradiation or the addition of a low-titer E1-, E3-deleted recombinant adenovirus resulted in up to a 100-fold increase in transgene product in the human, but not the mouse, cell lines. RA synovial tissues implanted subcutaneously in severe combined immunodeficiency (SCID) mice, which were subsequently infected with rAAV, showed marked increases in transgene expression when co-infected with adenovirus. To our knowledge, this is the first study to show that intact human synovial tissues can be transduced by rAAV, and it suggests that murine arthritis may not be an optimal model to study rAAV as a gene transfer vector. Further studies to elucidate the mechanisms limiting gene transduction in human synovium may allow optimization of this vector for the treatment of arthritis.

Key words Adeno-associated virus (AAV) · Arthritis · Gene therapy · Synovium

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Introduction

Autoimmune arthritides are leading causes of long-term disability in the United States. Rheumatoid arthritis (RA) affects approximately 1% of the population. The autoimmune basis of these diseases is well established, but the causes are unknown. The normal joint is lined with a 1–2-cell layer of synovial membrane. The inflammation in RA is characterized by the recruitment of immune cells, leading to massive thickening of the synovium accompanied by the release of inflammatory mediators, ultimately leading to the invasion and destruction of articular cartilage and bone.[1–3]

Present therapies, while partially effective in controlling symptoms and slowing the disease course, may not ultimately prevent disease progression. Thus, there is a great need for innovative therapeutic approaches for arthritis. Local overexpression of anti-inflammatory proteins by synoviocytes represents an attractive approach to treatment. Synoviocytes can be readily accessed by intraarticular injection, making them good targets for gene delivery. These cells have a low mitotic rate[4–6] and therefore are likely to express transduced genes for a considerable length of time, even if the transgene is episomally located.

The feasibility of direct in vivo gene transfer to synovium has been well demonstrated with adenoviral vectors in mice, rats, and rabbits.[7–16] However, adenovirus-mediated synovial gene transfer results in short-term gene expression which persists for 1–2 weeks only, and is accompanied by an antiviral inflammatory response.[7,9,10] It therefore represents an unlikely candidate vector for a chronic inflammatory

process, such as RA, in which long-term expression of therapeutic gene products will be required.

Recombinant adeno-associated virus (rAAV) has recently been shown to mediate very long-term gene transfer in a number of tissues. Its low immunogenicity makes this vector attractive for the treatment of chronic diseases. Furthermore, its lack of apparent pathogenicity in humans makes its safety profile compelling. rAAV is capable of transducing a variety of tissues, including skeletal muscle,[17,18] liver,[19] neurons,[20–23] and retina.[24]

In a recent study, we investigated the use of rAAV for in vivo gene transfer to mouse synovium.[25] Intraarticular injection of mouse knee joints resulted in the transduction of both periarticular and synovial lining, with the majority of transgene expression in the periarticular areas. The synovial lining consists of type A (macrophage-like) and type B (fibroblast-like) synoviocytes. The ability of rAAV to transduce fibroblasts is species-specific,[26] and therefore the poor transducibility of mouse synovial lining does not rule out the potential use of rAAV to target human synovium. In this study, the transduction efficiency of rAAV for human synovium was investigated.

Materials and methods

Mice

Male DBA/1J mice, 6–10 weeks of age, and female NOD.CB17-Prkdc SCID mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and housed under Institutional Animal Care and Use Committee (IACUC) approved conditions in the animal resource facility at the Children's Hospital Research Foundation, Cincinnati.

Recombinant AAV vectors

All rAAV constructs were derived from AAV2 and driven by the cytomegalovirus (CMV) promoter. rAAV(LacZ), encoding the *Escherichia coli* β-galactosidase (LacZ) gene, rAAV(mIL-4), encoding murine IL-4 cDNA, rAAV(mIL-10), encoding murine IL-10 cDNA, rAAV(eGFP), encoding the green fluorescent protein (GFP) cDNA, and rAAV(Luc), encoding firefly luciferase cDNA, were generated by either the 293/triple transfection or the B50/hybrid method.[27] Adenovirus helper functions were supplemented by pAdΔF6, a plasmid construct carrying all essential adenovirus helper genes.[27] Transfection was carried out using the standard calcium phosphate precipitation method. rAAV vector preps were purified by CsCl gradient centrifugation.[28] The genome titers of vector preps were determined by the real-time quantitative polymerase chain reaction (PCR) method,[27] whereas the transducing titers of vector preps were assayed on 84-31 cells as described elsewhere.[29] Virus was stored at −80°C in buffer containing 20 mM Tris pH 7.4, 1 mM $MgCl_2$, 150 mM NaCl, and 10% (v/v) glycerol.

Recombinant adenovirus

Ad(BglII) is an E1a-E3-deleted replication-defective adenovirus type-5 backbone vector lacking a transgene, and was generously provided by J.A. Bluestone and J.M. Leiden. Recombinant adenovirus was produced and propagated in 293 cells and purified by cesium chloride density centrifugation, as previously described.[30,31] Virus plaques were purified three times before the production of seed stocks, and their identities were confirmed by restriction endonuclease and DNA sequence analysis. Viral titers (particles per millilitre) were calculated by OD260 $\times$ 10^{12} following lysis of viral stocks in 0.1% SDS, 10 mM Tris-HCl (pH 7.4), and 1 mM EDTA at 56°C for 10 min. Virus was stored at −80°C in buffer containing 10 mM Tris pH 7.4, 1 mM $MgCl_2$, and 10% (v/v) glycerol.

Isolation of synoviocytes

Human RA and mouse collagen-induced arthritis (CIA) synovial tissues were finely chopped, washed with sterile PBS, resuspended in 4 mg/ml collagenase (Worthington Biochemical Corporation, NJ, USA) and incubated in a 37°C CO_2 humidified incubator for 17 h. After washing with phosphate-buffered saline (PBS), dissociated cells were resuspended in RPMI supplemented with 10% fetal calf serum, and adherent cells were passaged in culture for 1–2 weeks. Adherent cells lines were shown to have the phenotype of type B synoviocytes, as assessed by flow cytometry staining for CD90.

In vitro transduction and assays for IL-4 and IL-10

Cultured synoviocytes were plated at a density of 2 $\times$ 10^5 cells per 24-plate well for 48 h. Cultures were then infected with 1.0 $\times$ 10^4 viral particles per cell of rAAV(mIL-4) or rAAV(mIL-10). Some cultures were co-infected with 100 particles per cell of Ad(BglII). Some cultures were exposed to γ-irradiation from a ^{137}Cs source immediately prior to infection. In some experiments, a rat IgG2a anti-mouse IL-4 receptor α-chain monoclonal antibody (a gift from Dr. Fred Finkelman) was added to the cultures to block the consumption of IL-4. Titers of mIL-4 and mIL-10 in supernatants were determined by enzyme-linked immunosorbent assay (ELISA).[25]

In vivo transduction of RA synovium

Synovial tissue specimens from RA patients undergoing joint replacement surgery were cut into pieces of 1 mm^3. Five such fragments were engrafted subcutaneously in severe combined immunodeficiency (SCID) mice by forming a subcutaneous pocket over the back area and inserting the fragments. After allowing 7–10 days for engraftment, the RA tissues were visualized, and 100 μl rAAV and/or rAd was injected into the grafts. Control tissues were injected with buffer. The mice were killed 14 days later, and

engrafted synovial tissues were removed and snap-frozen for later analysis.

Luciferase assays

Human synovial tissue explants were placed in 0.5 ml Reporter Lysis Buffer (Promega, Madison, WI, USA) with 1 mM Ca^{2+}, homogenized with a tissue homogenizer (PowerGen 700, Fischer Scientific, Pittsburgh, PA, USA), incubated for 20 min at room temperature, and centrifuged for 10 min at 10 000 r.p.m. One hundred microlitres of the lysates were mixed with 100 µl Bright-Glo Luciferase Assay Reagent (Promega). Luciferase activity was measured with a luminescence counter (TopCount NXT Microplate Scintillation and Luminescence Counter, Packard BioScience, CT, USA), and expressed as relative light units normalized to total protein concentration determined using bovine serum albumin as a standard.

Staining for β-galactosidase transgene expression

Ten-micrometre cryosections were fixed in PBS + 1.25% glutaraldehyde at 4°C for 10 min. After washing three times in cold PBS, the sections were incubated overnight at 27°C in X-Gal solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.7 mg/ml X-Gal). The sections were washed three times in nanopure water, and counterstained with eosin.

Immunohistochemical staining for (GFP) transgene expression

Ten-micrometre cryosections were fixed for 10 min in 2% paraformaldehyde at room temperature, and endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide. The sections were incubated overnight in 10% nonfat dry milk to block nonspecific binding. The sections were incubated with rabbit anti-GFP polyclonal antibody (Chemicon, Temecula, CA, USA) for 1 h at room temperature, washed, and incubated with biotinylated goat antirabbit IgG (Vector Labs, Burlingame, CA, USA) for 30 min at room temperature. Following washing, the sections were incubated with Vectastatin ABC-Elite reagent (Vector Labs) followed by 3,3′-diaminobenzidine (DAB), with nickel chloride enhancement. The sections were counter-stained with nuclear fast red.

Results

Human and murine synoviocytes are poorly transduced by rAAV alone

The ability of rAAV to transduce fibroblasts is species-specific.[26] To determine the ability of rAAV to transduce human type B synoviocytes, fibroblast-like synovial cell lines were established from synovial tissues from three patients with RA undergoing joint replacement surgery, and compared with synoviocyte lines from the knee joints of two mice with CIA. These cell lines were infected with rAAV(mIL-10). At a titer of 10^4 particles of rAAV per cell, transgene expression in the mouse lines was close to the lower limits of detection, and expression in the human lines, while higher, was modest (Fig. 1). rAAV of serotypes 1 and 5 showed similar low-efficiency expression (data not shown).

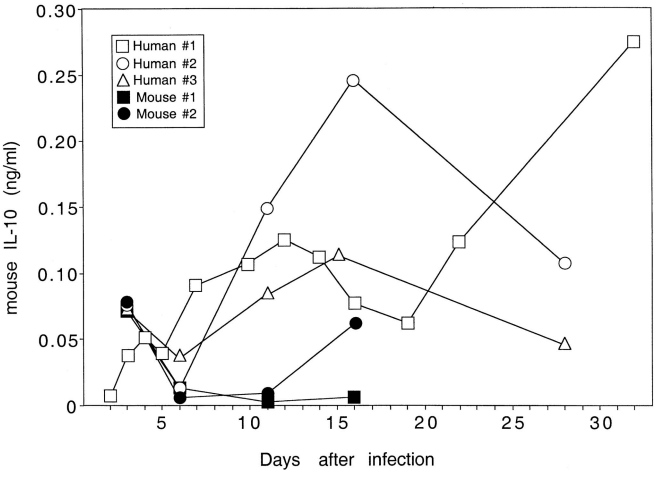

Fig. 1. Human and murine synoviocytes are poorly transduced by recombinant adeno-associated virus (rAAV) alone. Type B synovial cell lines from patients with rheumatoid arthritis (RA) or mice with collagen-induced arthritis (CIA) were grown in 24-well plates and infected with rAAV(mIL-10). mIL-10 in the supernatants was assayed on the days indicated and represents the concentration of mIL-10 secreted over a 24-h period

Adenovirus or genotoxic stress markedly enhance rAAV-mediated transduction of human, but not murine, synoviocytes

Transduction by rAAV is a multistep process requiring infection of the cell, migration of the viral genome to the nucleus, and second strand DNA synthesis of the viral genome, which is packaged as a single strand. To address the possibility that second-strand synthesis was a limiting factor in the transduction of synoviocytes, cell lines were co-infected with 100 particles per cell of Ad(BglII), a replication incompetent adenovirus lacking a transgene. Ad(BglII) provides adenoviral E4 genes which function to induce second-strand synthesis. A marked increase in gene expression was observed in the human, but not the mouse, lines (Fig. 2), suggesting that second-strand synthesis is a rate-limiting factor in the transduction of human type B synoviocytes. The lack of an increase in the mouse lines was not due to the lack of tropism of adenovirus for murine fibroblasts, as they are permissive.[32–34] The enhanced transduction of human synoviocytes in the presence of adenovirus was not specific to the mIL-10 transgene, as it was also observed following transduction with rAAV(mIL-4) (Fig.

3). Genotoxic stress, in the form of γ-irradiation, also enhanced rAAV-mediated transduction of human synoviocytes (Fig. 3). To ensure that the lower amount of transgene product in the supernatants of mouse synoviocyte lines was not due to binding of the mouse cytokines to cell surface receptors, experiments were repeated in the presence of blocking antibody to mouse IL-4 receptor.[35] As shown in Fig. 4, minimal transgene product was detected in supernatants of mouse lines, even in the presence of antibody to IL-4 receptor.

Adenovirus enhances in vivo transduction of human RA synovium

In order to study the effects of rAAV on human synovium, we adapted a model in which fragments of human synovial tissue are implanted into mice with SCID, which has been used successfully by several groups to study aspects of the pathogenesis of RA.[36,37] Following subcutaneous implantation into SCID mice, RA synovial tissues revascularize and maintain their inflammatory phenotype for at least 2 months. An example is shown in Fig. 5A.

RA synovial tissues were implanted into SCID mice. After a 2-week engraftment period, the implants were injected with 10^{10} particles of rAAV(luc), encoding the luciferase reporter gene, with or without the addition of 10^{8} particles of Ad(BglII). The tissues were removed 2 weeks later and analyzed for luciferase expression. As with our in vitro observations, minimal transgene expression was detectable with rAAV alone, but a marked increase in expression was observed following co-infection with adenovirus (Fig. 5B). Enhanced rAAV-mediated transduction in the presence of helper adenovirus was also observed in tissues injected with rAAV(*LacZ*) (Fig. 5C,D) and rAAV(GFP) (Fig. 5E,F).

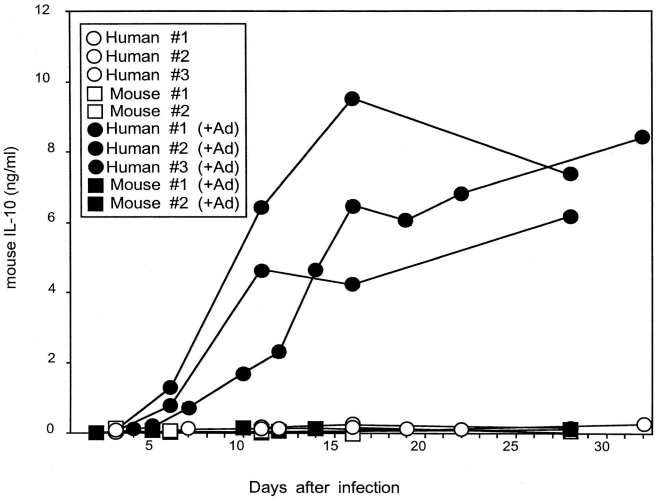

Fig. 2. Adenovirus markedly enhances rAAV-mediated transduction of human, but not murine, synoviocytes. Type B synovial cell lines were grown in 24-well plates and infected with rAAV(mIL-10) with or without the addition of 100 particles/cell of the backbone adenovirus, Ad(BglII). mIL-10 in the supernatants was assayed on the days indicated and represents the concentration of mIL-10 secreted over a 24-h period

Discussion

There have been a number of reports on the use of rAAV in models of arthritis. These studies have shown somewhat conflicting findings, although a pattern is beginning to emerge. Consistent with studies demonstrating that mouse fibroblasts are not permissive for rAAV, mouse synovium appears to be relatively resistant to transduction by rAAV. The ability of rAAV to transduce fibroblasts appears to be species-specific,[26] with mouse fibroblasts showing poor transduction, even though they express rAAV receptors and are permissive for second-strand synthesis.[26,38] This appears to be due to impaired trafficking of virus to the nucleus. Our initial report on rAAV-mediated in vivo transduction of mouse synovium showed the transduction of both synovial lining and subsynovial muscle tissue.[25] We have subsequently performed a more detailed analysis of cellular localization which showed that the majority of transduction

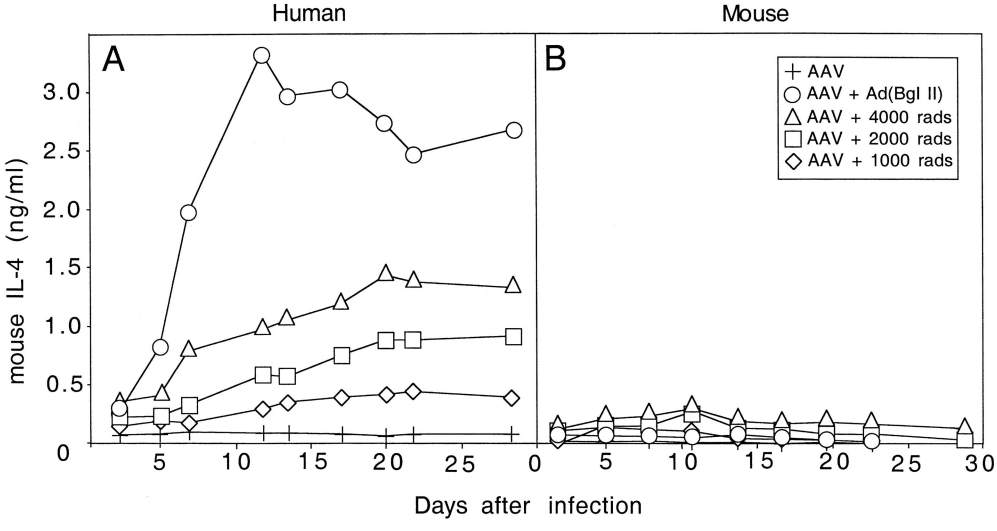

Fig. 3. Adenovirus and γ-irradiation markedly enhance rAAV-mediated transduction of human (**A**), but not murine (**B**), synoviocytes. Type B synovial cell lines were grown in 24-well plates and infected with rAAV(mIL-4) with or without the addition of 100 particles/cell of the backbone adenovirus, Ad(BglII). mIL-4 in the supernatants was assayed on the days indicated and represents the concentration of mIL-4 secreted over a 24-h period

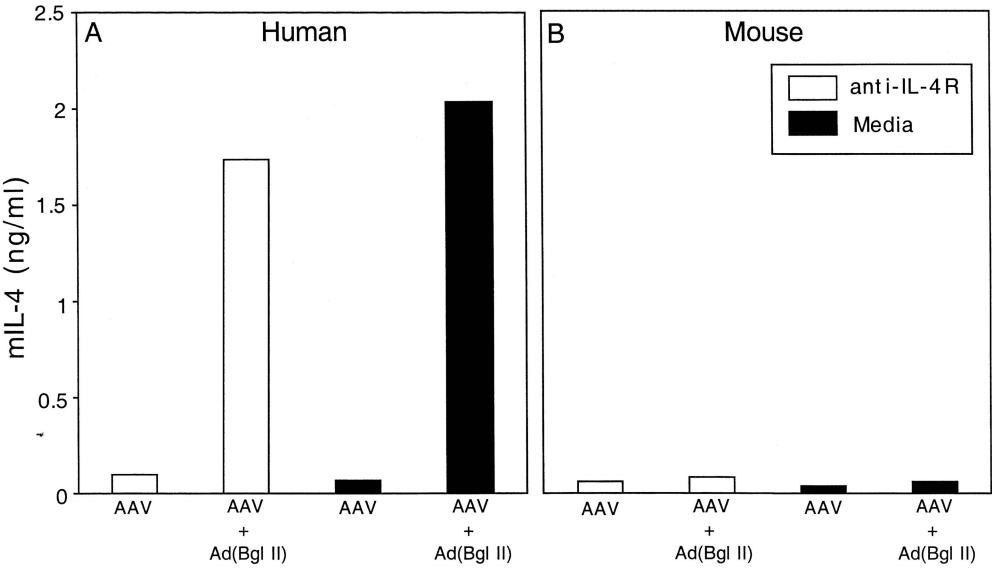

Fig. 4. Poor transducibility of mouse (**B**) compared with human (**A**) synoviocytes is not due to the consumption of mIL-4 by IL-4 receptor-bearing cells. Type B synovial cell lines were grown in 24-well plates and infected with rAAV(mIL-4) with or without the addition of 100 particles/cell of the backbone adenovirus, Ad(BglII). Where indicated, cells were cultured in the continuous presence of 10 μg/ml anti-IL-4 receptor antibody. mIL-4 in the supernatants was assayed on day 14, and the data represent the concentration of mIL-4 secreted over a 24-h period

is in the muscle, with only limited transduction of the synovium. Similar results have also been reported by Cottard et al.[39] and Zhang et al.[40] This is a critical point, as the mouse knee joint has a capacity of only 5 μl, and unless injections are performed under stringent conditions, it is technically challenging to target the synovium specifically.[7] Goater et al. reported that, as in the above observations, synovium of healthy mice showed limited transduction by rAAV. However, they found a short-term increase in transgene expression in arthritic mice transgenic for human TNF-α. They also observed increased transduction of mouse primary fibroblast-like synoviocytes in the presence of TNF. We did not observe increased transduction in arthritic mice, but DBA1 mice with CIA do not express a large amount of TNF.[41] Thus, the findings of Goater et al. may represent a specific effect of TNF on rAAV-mediated transduction of mouse synoviocytes. Goater et al. also reported increased in vitro transduction in mouse synoviocytes exposed to UV or γ-irradiation, in contrast to the findings presented here. However, they only reported the percentage increase over baseline expression and not the absolute amount of transgene product. We observed increases in transgene expression in mouse fibroblast-like synoviocytes in the presence of γ-irrradiation or adenovirus, as can be seen in Fig. 3, but the absolute quantity of product was quite modest compared with what we observed with the human lines.

To our knowledge, our study is the first to demonstrate that human synovial tissues can be transduced by rAAV in vivo. We observed transduction of human synovium both in vitro and in vivo, which was enhanced in the presence of adenovirus or γ-irradiation, and was consistent with other studies showing that human fibroblast transduction can be increased using agents that enhance second-strand synthesis.[42] Zhang et al.[40] recently reported that human RA synovial fibroblasts could be transduced by rAAV in vitro, which supports our findings with human RA synoviocytes.They reported higher levels of transgene expression than those observed in this study. This may reflect patient-to-patient variability, as they suggest in their report, or possibly differences related to the transgenes.

Taken together, the results presented here, in addition to previously published studies, suggest that mouse synovium is poorly permissive for rAAV. It might be possible to circumvent this by exposure to TNF, although this requires further validation. Human RA synoviocytes, in contrast, are permissive, and transgene expression can be markedly enhanced by the use of helper factors. This might be advantageous, as it suggests the possibility of regulating expression in synovium. Given the chronic nature of RA and related diseases, an optimal gene transfer vector would most likely need to induce long-term transgene expression where gene regulation assumes increased importance. While the results reported here suggest that rAAV may not be an ideal vector for human arthritis, there may be means to optimize its use in this setting. A better understanding of the mechanism by which adenovirus enhances rAAV-mediated transduction might allow for improved methods of delivering rAAV to synoviocytes. In addition, other approaches to enhancing rAAV-mediated transduction can be explored. For instance, second-strand synthesis can be enhanced with UV or γ-irradiation.[43–45] Local low-dose irradiation to the joint might be acceptable in humans if done infrequently. Radiation synovectomy has been used as a treatment for RA, although it has fallen out of favor because the synovitis recurs. An alternative clinical approach would be to deliver UV irradiation, as has recently been tested in a pilot study of 66 patients who were given oral 8-methoxypsoralen followed by UV light delivered by an arthroscope.[46] Further studies to elucidate the mechanisms limiting gene transduction in human synovium may allow the optimization of rAAV for the treatment of arthritis.

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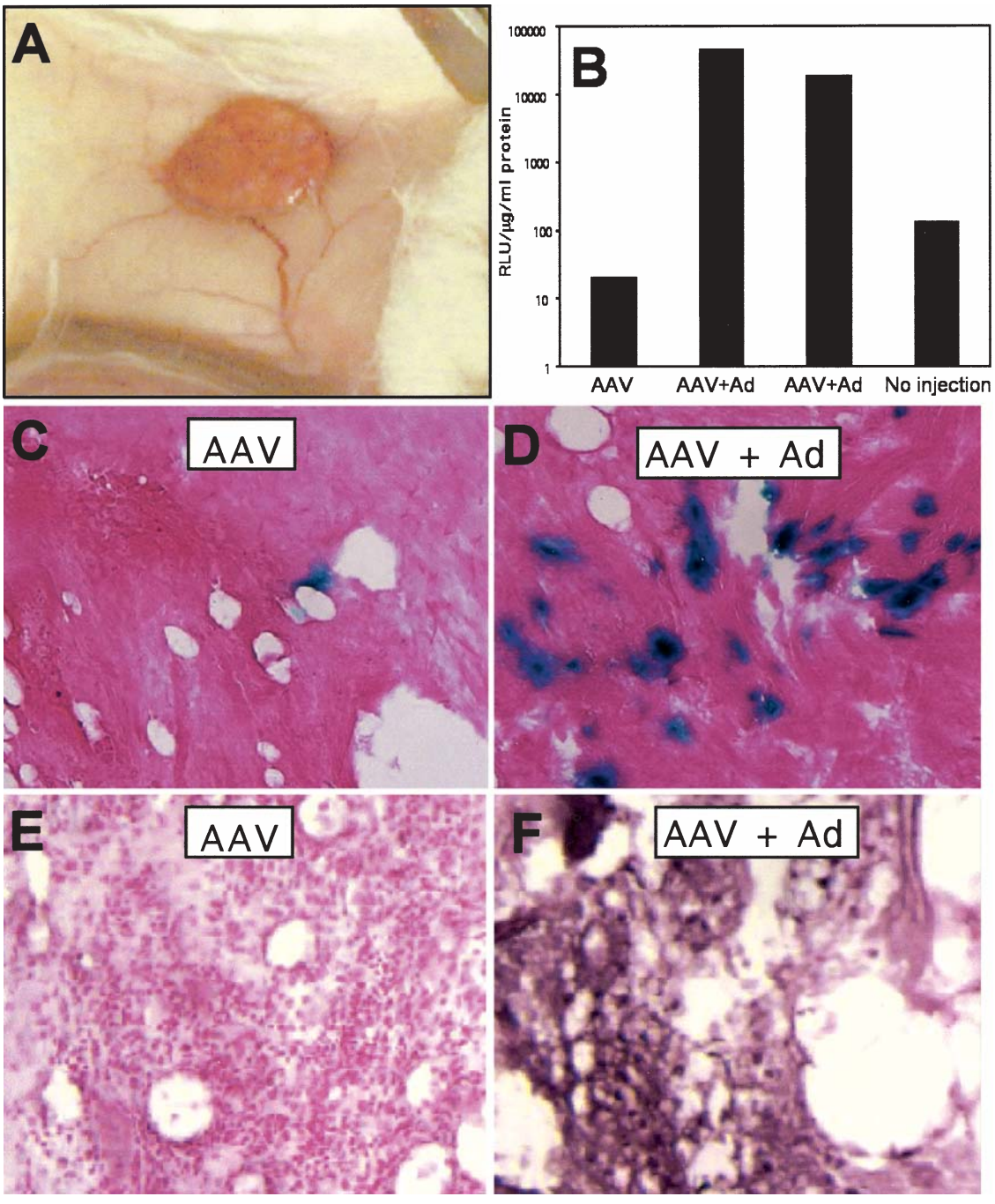

Fig. 5. Adenovirus enhances in vivo transduction of human RA synovium. Human RA synovial tissues were implanted subcutaneously on the backs of severe combined immunodeficiency (SCID) mice and allowed to engraft over a 2-week period, during which time the implants developed a vascular supply to the mouse (**A**). After 2 weeks, the implants were injected with rAAV(luc) (**B**), rAAV(lacZ) (**C**,**D**), or rAAV(GFP) (**E**,**F**) with or without Ad(BglII). Two weeks later, RA synovium was removed and assayed for luciferase content (**B**), or stained with X-gal (**C**,**D**) or with antibody to GFP (**E**,**F**)

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