A New Strategy to Deliver Synthetic Protein Drugs: Self-reproducible Biologics Using Minicircles

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Biologics are the most successful drugs used in anticytokine therapy. However, they remain partially unsuccessful because of the elevated cost of their synthesis and purification. Development of novel biologics has also been hampered by the high cost. Biologics are made of protein components; thus, theoretically, they can be produced in vivo. Here we tried to invent a novel strategy to allow the production of synthetic drugs in vivo by the host itself. The recombinant minicircles encoding etanercept or tocilizumab, which are synthesized currently by pharmaceutical companies, were injected intravenously into animal models. Self-reproduced etanercept and tocilizumab were detected in the serum of mice. Moreover, arthritis subsided in mice that were injected with minicircle vectors carrying biologics. Self-reproducible biologics need neither factory facilities for drug production nor clinical processes, such as frequent drug injection. Although this novel strategy is in its very early conceptual stage, it seems to represent a potential alternative method for the delivery of biologics.

Biologics are modern drugs that are made of protein components. Biologics have been very successful in the treatment of cancer and autoimmune diseases that were previously difficult to treat. In the field of rheumatology in particular, etanercept, which comprised the tumor necrosis factor (TNF) receptor2-Fc fusion protein, yielded a marked improvement of the symptoms and signs of rheumatoid arthritis (RA) as TNFα was identified as a main player in the pathophysiology of RA. Other than TNFα, IL-6 plays a critical role in inflammation and became a major target in the rush of biologics development. The anti-IL-6R monoclonal antibody (tocilizumab, Actemra®) was also successfully launched in clinical practice.

As biologics have become widely used in clinical practice, it seems clear that biologics opened a new era in the development of novel drugs. Because signal blockade using biologics such as antibodies and soluble receptors can be achieved more easily compared with chemical drugs, target selection enables the development of new drugs. Numerous proteins, including cytokines and chemokines, that play major roles in the pathogenesis of diverse diseases can be considered as targets for biologics. However, it is almost impossible to develop new biologics at the small scale because of the high cost of their purification and functional characterization. Thus, a new approach to the development and evaluation of novel biologics with a relatively low cost and requiring a short time is needed.

Furthermore, biologics also pose problems from a socioeconomic point of view. For example, TNF inhibitors are classified as the most expensive drugs. They cost an average of $20,000/month and need to be used for long periods. The high cost of TNF inhibitors is partly explained by their sophisticated manufacturing process, which must fit the strict guidelines for biological processes that are used in human clinical applications. These hurdles may prevent the production of the drug by the pharmaceutical industry within a reasonable price range.

Here, we propose the minicircle vector system as a solution for the high cost of the development and production of biologics. Minicircle vectors (minicircles) are protein-expressing vectors in which the bacterial backbone has been removed. As bacterial-backbone parts are not necessary for gene expression and may induce an immune response in mammalian cells, minicircles are considered as ideal plasmids for transgene expression in vitro and in vivo. The size of minicircles, which is smaller than that of conventional plasmids, increases the possibility of delivering these vectors into cells. Moreover, it has been reported that minicircles escape gene silencing and show sustained expression because of the unique methylation patterns found in these vectors.
Recently, several groups have used minicircles as tools for transgenic expression in vivo based on these advantages. Some of them delivered minicircles encoding natural proteins, such as the hypoxia-inducible factor-1 alpha (HIF-1α), alpha-l-iduronidase (IDUA), and interferon gamma (IFNγ), into animals to examine the efficiency of minicircles for use in gene therapy.

Minicircles encoding shRNAs or microRNAs have also been reported as good tools for gene therapy. Moreover, Adamopoulos et al. developed an arthritis mouse model using minicircles encoding IL-23, which is one of the pro-inflammatory cytokines. This research has shown that minicircles enable the expression of transgenes using the protein-synthesis system of hosts.

In this paper, we successfully invented a novel strategy of drug delivery without injection of the actual therapeutic product. We generated vectors enclosing the nucleotide sequence of etanercept and tocilizumab based on the backbone of a minicircle structure. Our data showed that the intravenous injection of minicircles encoding synthetic drug sequences induced the in vivo production of synthetic protein drugs. We confirmed that the self-reproduced drug was functionally active and relevant in arthritic mice. The minicircle system will be useful for the development of novel drugs, even in small-size laboratories, as it allows skipping complex processes such as the synthesis and purification of protein drugs. Furthermore, although there are still safety limitations and efficacy hurdles to overcome, the self-reproducible strategy may be applicable to the treatment of patients in the future, which might help overcome the shortage of biologics based on cost, monopolized production, etc., as mentioned above.

Results

Generation of minicircle vectors encoding anti-IL-6R or sTNFR2-Fc. As biologics consist of peptides, we predicted that they can be expressed in a host via the injection of minicircles. To confirm this hypothesis, we chose drugs with an established effect: tocilizumab (anti-IL-6R) and etanercept (sTNFR2-Fc). To generate more efficient plasmids encoding sTNFR2-Fc (pp_sTNFR2-Fc), sTNFR2-Fc DNA sequences were subcloned into the parental plasmid pMC.CMV-MCS-EF1-GFP-SV40polyA (pp_mock) (Fig. 1a). As the anti-IL-6R is an antibody molecule, the heavy-chain and light-chain DNA sequences of the anti-IL-6R antibody were subcloned into pp_mock separately (pp_anti-IL-6R-HC and pp_anti-IL-6R-LC). We predicted that the transfection of both plasmids into cells would lead to the construction of the intact form of anti-IL-6R antibodies. Each insert was subcloned downstream of the CMV promoter, whereas green fluorescence protein (GFP) sequences were placed under the control of the EF1 promoter, which facilitates the detection of the expression of the insert and its localization in cells or tissues (Fig. 1a). After incubation with L-arabinose, minicircle vectors were produced, and the remaining bacterial sequences were degraded (Fig. 1b). Purified minicircle vectors were used for further experiments. Before generating minicircles from these newly constructed parental plasmids, we confirmed that the expressible DNA sequences intended for subcloning were contained in the parental plasmids via DNA sequencing. Table S1 provides the peptide sequences of protein drugs. We confirmed that the parental plasmids encoding protein drugs were properly converted to minicircle vectors after treatment with L-arabinose. After digestion with XbaI and BamHI, the DNA fragments of interest were present in the minicircle vectors (Supplementary Fig. S1 online).
compared with control samples (Fig. 2a). As GFP expression is not the perfect marker of the expression of the inserted drug sequences, we designed an enzyme-linked immunosorbent assay (ELISA) to detect the expression and secretion of protein drugs from minicircle-transfected cells. The culture media of transfected cells were collected, and the levels of the anti-IL-6R and sTNFR2-Fc proteins were assessed by ELISA. The results of these experiments showed that minicircles carrying the DNA sequences of the protein drug yielded efficient expression of each drug (Fig. 2b and 2c).

To examine whether the minicircle-derived drugs functioned properly, we administered the conditioned media of HEK293T cells transfected with mc_sTNFR2-Fc or mc_anti-IL-6R to fibroblast-like synoviocytes (FLS) of RA patients (RA-FLS) in conditioned media containing proinflammatory cytokines. Because TNF is believed to play a pivotal role in the uncontrolled proliferation of FLS in RA2, it was estimated that mc_sTNFR2-Fc-derived drug would block the proliferation of these cells. We found that the conditioned media of mc_sTNFR2-Fc-transfected cells prevented the proliferation of FLS, which indicated that the mc_sTNFR2-Fc-derived drug functioned properly (Fig. 2d).

On the other hand, the conditioned media of mc_anti-IL-6R-transfected cells did not prevent the proliferation of FLS. This might be because IL-6 signaling is less involved in the proliferation of fibroblasts than TNF signaling13,15. IL-6 is an important cytokine for the migration of fibroblasts16; therefore, we performed scratch assays to determine the function of mc_anti-IL-6R-derived drugs in vitro (Fig. 2e and f). When RA-FLS were incubated with IL-6, sIL-6R, and TNFα, the migration of these cells was higher than that of negative control cells. Cell migration was not blocked by treatment with the conditioned media of HEK293T cells transfected with mc_mock. However, the conditioned media of HEK293T cells transfected with mc_anti-IL-6R or mc_sTNFR2-Fc inhibited migration of cells in a dose-dependent manner. These data showed that protein drugs (sTNFR2 or anti-IL-6R) carried by minicircles could be expressed and functioned in vitro.

Amelioration of experimental arthritis by minicircles encoding anti-IL-6R or sTNFR2-Fc. To confirm that minicircles can be delivered and expressed in vivo, we intravenously injected minicircles lacking an insert but containing GFP into mice using a hydrodynamic procedure and investigated GFP expression 48 h after injection using a Maestro multispectral system. GFP signals were detected from minicircle-injected mice, whereas no signal was detected from normal saline-injected mice, which indicated that the injection of minicircles can induce the expression of minicircles in vivo (Supplementary Fig. S2). The fluorescence-microscopy images of cryosectioned tissues from these mice showed that GFP originating from minicircles was expressed in at least the liver, stomach, and large intestine (Supplementary Fig. S3). Other tissues, including the spleen, kidney, brain, lymph nodes, and lung, were also investigated; however, the autofluorescence signals were too intense to allow the differentiation of genuine signals (data not shown). These data indicate that our minicircle vectors qualified as vehicles for the expression of a target protein in vivo.

To examine the effect of minicircles encoding protein drugs in vivo, mc_anti-IL-6R and mc_sTNFR2-Fc were delivered to collagen-induced arthritis (CIA) mice which represent an experimental mouse model of RA via a hydrodynamic procedure. The disease severity score was monitored. As anti-IL-6R and sTNFR2-Fc are the representative drugs for RA, we expected that arthritis would subside if minicircle-derived protein drugs were expressed appropriately using the biological systems of the animals. We observed that both mc_anti-IL-6R-injected mice and mc_sTNFR2-Fc-injected mice showed amelioration of arthritis, as demonstrated by a reduced arthritis severity score (Fig. 3a). The arthritis-suppressive effects of mc_sTNFR2-Fc were sustained to a later stage than were those of mc_anti-IL-6R. In addition, the disease incidence of mice injected with mc_anti-IL-6R or mc_sTNFR2-Fc tended to increase slower than that of CIA group (Fig. 3b).

The extent of joint destruction and inflammation was examined by H&E staining of sections of the hind paw (Fig. 3c). Although synovial hyperplasia, severe infiltration, and bone destruction were present in the joints of CIA mice, the joints of mice injected with minicircles encoding protein drugs were largely spared. The inflammation states of joints were scored based on H&E staining results; the scores of mice injected with protein-drug-encoding minicircles were significantly lower than those of mice in the CIA group (Fig. 3d). We performed safranin O staining and toluidine blue staining to measure the extent of cartilage damage in joints. The results of these experiments indicated that minicircle-encoded drugs prevented the loss of cartilage in this arthritis mouse model (Fig. 3c). The joint-destruction score based on the safranin O and toluidine blue staining showed that the joints of mc_sTNFR2-Fc-injected mice were ~86% more preserved than were those of CIA mice (Fig. 3e). mc_anti-IL-6R injection also had positive effects regarding the prevention of joint destruction in CIA, although the effect was milder than that of mc_sTNFR2-Fc. Because cartilage damage, pannus formation, and bone erosion progress over time after the onset of arthritis, mc_anti-IL-6R may hamper those symptoms more efficiently than expected from the arthritis severity score. These data suggest that the injection of minicircles encoding protein drugs improves arthritis in mice, regardless of the form of the protein drugs: soluble receptors were effective, and even a more complicated form, an antibody, also had an effect.

**Biologics expressed in vivo using the animals’ own protein-synthesis system.** To confirm that the alleviation of the disease state observed was caused by the protein drugs that were expressed from minicircles, we collected the serum of mice and evaluated the expression levels of drugs in the serum using ELISA (Fig. 4a and b). Significant amounts of anti-IL-6R and sTNFR2-Fc were detected in each of the groups of mice injected with mc_anti-IL-6R and mc_sTNFR2-Fc, respectively.

We also tried to confirm the existence of cells expressing the target proteins derived from minicircles in vivo via immunohistochemical staining with anti-GFP antibody. As hydrodynamic delivery is a method that increases the permeability of endothelial cells in capillaries, with instantly elevated pressure17,18, the liver is the most common site at which gene transfer occurs, as it contains a vast amount of capillaries19-21. Therefore, first we assessed the presence of GFP-positive cells in the liver of mice (Fig. 5). As shown in the figure, GFP-positive cells were detected in the livers of mice injected with minicircles, but not in those from WT and CIA mice injected only with normal saline.

We predicted that drug-expressing cells would be located in the synovium of CIA mice injected with minicircles, as there is a considerable amount of blood vessels in this structure in CIA mice because of neoangiogenesis, which is one of the characteristics of arthritis22-24. Immunohistochemical staining of joint tissues with anti-GFP antibody showed that a large amount of GFP-positive cells were present in the synovium of mice injected with minicircles (Fig. 6, top and middle rows). Interestingly, significant amounts of minicircle-expressing cells were also detected in cartilages (which do not have blood vessels) of CIA mice injected with minicircles (Fig. 6, top and middle rows). As it has been reported that FLS can be differentiated into chondrocytes25, these minicircle-expressing cells located in cartilage may have originated from minicircle-expressing FLS. These data suggest that minicircles encoding the protein drugs sTNFR2-Fc and anti-IL-6R ameliorated the symptoms of experimental arthritis, and protein drugs were expressed in vivo using the animals’ own protein-synthesis system.
Figure 2 | Expression of protein drugs derived from minicircles and their function in vitro. (a) Fluorescence images showing the expression of the GFP encoded in each minicircle. HEK293T cells were transfected with mc_mock, mc_anti-IL-6R, or mc_sTNFR-Fc, and GFP expression was analyzed by fluorescence microscopy 24 h post-transfection. HEK293T cells which were not transfected with minicircles (Ctrl) were used as a control group. Scale bars: 500 μm. (b) Concentration of the anti-IL-6R antibody present in conditioned media of HEK293T cells transfected with mc_mock or mc_anti-IL-6R (mean ± s.e.m). The conditioned media were obtained 24 h post-transfection and analyzed by ELISA. (c) Concentration of sTNFR2-Fc in the conditioned media of HEK293T cells transfected with mc_mock or mc_sTNFR2-Fc (mean ± s.e.m). The conditioned media were removed 24 h post-transfection and analyzed by ELISA. (d) Anti-proliferative effect of the protein drugs originated from mc_sTNFR2-Fc on FLS of RA patients (RA-FLS). HEK293T cells were transfected with mc_mock, mc_anti-IL-6R or mc_sTNFR2-Fc, and the conditioned media were collected 24 h after the transfection. RA-FLS were treated with the conditioned media or with PBS (as a negative control). Some of the cells were incubated with IL-6, IL-6R, and TNFα for 72 h. The proliferation rate was evaluated using a CCK-8 assay kit (mean ± s.e.m). (e) Anti-migration effects of drugs derived from mc_anti-IL-6R or mc_sTNFR2. When RA-FLS had grown to confluency, the monolayer was scratched with a sterile pipette tip. Cells were treated with conditioned media (250 μL or 500 μL) of HEK293T cells transfected with minicircles. Phase-contrast microscopy images were acquired at 0 h and 18 h after wounds were created. (f) Percentage of invaded area. The tendency to migrate was calculated according to the following equation: percentage of invaded area = (1 - (wounded area at 18 h)/(wounded area at 0 h)) × 100. Mean ± s.e.m is presented. All results are representative of at least three independent experiments. *P values were obtained using the Student's t test (*, P < 0.05; **, P < 0.01).
Figure 3 | Amelioration of arthritis by minicircles encoding the anti-IL-6R antibody or sTNFR2-Fc. (a) Arthritis severity score of normal and CIA mice that were injected intravenously with vehicle (normal saline), mc_anti-IL-6R, or mc_sTNFR-Fc. Results represent the mean ± s.e.m. (b) Disease incidence. The incidence of arthritis was calculated as a percentage of mice of which one or more paws were swollen among all mice in the group. (c) Histological analysis of the hind paws of wild-type (WT) mice, CIA mice, and CIA mice injected with mc_anti-IL-6R or mc_sTNFR-Fc by H&E, safranin O, and toluidine blue staining. The black boxes on H&E images designate areas with safranin O and toluidine blue staining (images shown below). Scale bars: 500 μm in the H&E image and 200 μm in the safranin O and toluidine blue images. (d) Inflammation score of hind paws evaluated based on the histological analysis. The score reflects the extent of synovial hyperplasia and infiltration of leukocytes detected in the H&E staining shown in (b). (e) Joint-destruction score of hind limbs calculated based on the safranin O and toluidine blue staining results. The score represents the extent of pannus formation and erosion of cartilage. P values were obtained by Student’s t test (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
Longevity of biologics derived from minicircles in vivo. To investigate how long minicircle-affected cells exist in vivo, mc_mock was delivered to DBA1/J mice, and the livers of these mice, which were obtained at 5, 10, and 15 days after the injection, were analyzed by immunohistochemical staining with an anti-GFP antibody (Fig. 7a). While no signals were detected in any tissues of normal saline-injected mice (NS), there were positive signals in the tissues of minicircle-injected mice stained with an anti-GFP antibody. GFP-
positive cells were present at least 15 days after the injection, although the number of these cells was decreased at this time-point.

To examine how long the biologics were present in vivo, we collected the serum of mice that had been injected with mc_anti-IL-6R or mc_sTNFR2-Fc and performed an ELISA to evaluate the concentration of biologics in the serum (Fig. 7b and c). Anti-IL-6R antibody was detected at 5 days after the injection, and its concentration was slightly increased at 15 days after the injection. A significant amount of anti-IL-6R antibody was detected even at 30 days after the injection (Fig. 7b). Meanwhile, sTNFR2-Fc was detected at 5 days after the injection, and its concentration was greatly decreased at 10 days after the injection. Finally, sTNFR-Fc was not detected at 15 days after the injection (Fig. 7b). These data show that one injection with minicircles carrying biologics generated enough biologics for sTNFR2-Fc and anti-IL-6R antibody to be detected for about 10 days and more than 30 days, respectively.

Discussion

In this study, we invented a method to deliver artificial vectors encoding synthetic drugs in the form of a receptor–Fc fusion and a monoclonal antibody. This method is similar to conventional gene therapy using viral vectors, plasmids, etc. Kim et al. reported that injection of an expression plasmid encoding sTNFR2-Fc into the muscle of CIA mice effectively causes the disease to subside27. Although this previous study showed that biologics can be introduced as a gene, the delivery system used was in vivo electroporation,
which cannot be applied to human systemic diseases such as RA. Minicircles are useful for gene therapy because of the high efficiency with which they can be introduced in vivo via i.v. injection; therefore, minicircles can be used to effectively deliver plasmids into animals\textsuperscript{14}. Various attempts have been made to treat diseases by delivering minicircles encoding natural inhibitory molecules\textsuperscript{28,29}, such as HIF-

**Figure 7 | Longevity of minicircle-affected cells in livers and of biologics in serum.** (a) Results of immunohistochemical staining of the livers of WT mice injected with normal saline (NS) or mc_mock. At 5, 10, and 15 days after the injection, livers were removed from mice and analyzed by immunohistochemical staining with IgG or an anti-GFP antibody. Scale bars in the upper and bottom rows: 100 \( \mu \)m. (b) Concentration of anti-IL-6R antibodies in the serum of WT mice injected with mc_mock or mc_anti-IL-6R (mean ± s.e.m). At 5, 15, and 30 days after the injection, venous blood was collected from mice and the serum was analyzed by ELISA. (c) Concentration of sTNFR2-Fc proteins in the serum of WT mice injected with mc_mock or mc_sTNFR2-Fc (mean ± s.e.m). Venous blood was obtained from mice at 5, 10, and 15 days after the injection and the serum was analyzed by ELISA.
We verified the actual production and the functions of biologics from the minicircles in vitro and in vivo. HEK293T cells were transfected with the minicircles encoding biologics (Fig. 2). sTNFR-Fc and anti-IL-6R were produced and detected by ELISA in conditioned media of HEK293T cells (Fig. 2b and 2c). As expected, the biologics produced by minicircle transfection exhibited active biological functions, such as antiproliferative effect and anti-migration effect (Fig. 2d–f). To examine the effects of minicircles encoding biologics in vivo, articular mice were injected with the minicircles. The expressed biologics attenuated arthritis in mice (Fig. 3). sTNFR-Fc and anti-IL-6R were present in the serum of mice (Fig. 4). Their suppressive effect on arthritis was maintained for more than 3 weeks.

In this study, we showed that minicircles encoding a soluble receptor or an antibody could sufficiently express the encoded protein to ameliorate disease in vivo. However, the efficiency with which disease was ameliorated differed between these two forms. The arthritis severity scores of mice injected with mc_sTNFR2-Fc were lower than those of vehicle-treated CIA mice until 51 days after the injection. By contrast, the scores of mice injected with mc_anti-IL-6R were lower than those of vehicle-treated CIA mice only at an early stage after the injection (Fig. 3a; day 37–45), although the concentration of anti-IL-6R antibody in serum on a given day was higher than that of sTNFR2-Fc on the same day (Fig. 4). However, the pharmacodynamics of these two biologics were not considered, meaning we cannot affirm that disease is ameliorated less efficiently by anti-IL-6R antibody than by sTNFR2-Fc. Moreover, IL-6R signaling is reportedly only involved in the early stage of arthritis models20,21, inferring that the anti-arthritis effects of the anti-IL-6R antibody are restricted to the early stage of the disease. If this minicircle system is used to compare the efficiencies of biologics, the pharmacodynamics of the biologics and the level of the target protein in the pathogenesis of the disease should be carefully measured.

We used an expression vector encoding both GFP and biologics in which GFP expression was driven by an independent promoter (Fig. 1a). Unlike biologics, the GFP protein is not soluble and is not secreted. The detection of GFP expression in some cells or organs showed that those cells or organs were transfected with minicircles and expressed the GFP protein and biologics successfully. GFP expression implied that the cells or organs which expressed it were the source of secreted biologics. Our data showed that the liver expressed the largest amount of the GFP protein (Fig. 5), as demonstrated previously by other researchers17,18. Based on these data, we suppose that hepatocyte is one of the major cells to generate minicircle-derived biologics.

Interestingly, GFP expression was also detected in the swollen ankles of mice with induced arthritis (Fig. 6). To introduce the minicircle, we used hydrodynamic tail-vein injection. Hydrodynamic gene delivery uses the hydrodynamic force generated by the pressurized injection of a large volume of DNA solution into the blood vessel; therefore, it permeabilizes the capillary endothelium and leads to the formation of pores in the plasma membrane of the surrounding parenchyma cells20–22. It is known that angiogenesis contributes to the development and maintenance of RA24. And the hypoxic state observed in RA joint suggests that the formation of new blood vessels, angiogenesis, in the pannus is driven by the hypoxia-induced expression of VEGF25. It is possible that minicircles were transfected into the inflamed synovial tissues through the increase in the number of new blood vessels in the RA condition. The use of minicircle-encoded secreted biologics via hydrodynamic gene delivery may represent an advantage regarding the transport of these agents into the target cell or organ in a range of angiogenesis-associated disease states, such as malignancies, retinal neovascularization, and psoriasis.

When considering this minicircle system as a treatment tool for patients, several limitations need to be overcome. We expect that minicircles would express genes for longer than can be achieved using previously published methods without being being integrated into the host genome. In our experiments, GFP-positive cells were found in the livers of mice at 15 days after injection with mc_mock (Fig. 7a). Moreover, the concentration of anti-IL-6R antibody was significantly high in the serum of mc_anti-IL-6R-injected mice even at 30 days after the injection (Fig. 7b). A drug that is sustained within the system usually favors treatment efficacy, but may be unfavorable in particular conditions if its biological function ceases suddenly. For example, clinicians will discontinue the use of biologics if the patient develops a serious infection or malignancy. For clinical applications, the minicircle strategy needs to be fine-tuned and its working period warrants adjustment.

The injection method and hepatotoxicity represent another safety issue. Minicircles work especially well if they were infused intravenously via high hydropressure and using a large volume. However, hydrodynamic delivery has been reported to elevate liver enzymes in blood22. This phenomenon is partially explained by the fact that voluminous infusion affects the hepatic sinus and increases hepatic pressure. Thus, minicircle infusion should be restricted in patients with liver problems. As it is recently suggested that electrotransfer is efficient method to transfer minicircles for tissue-targeted gene delivery23, electrotransfer can be considered as an alternative.

The in vivo expression system using minicircles still needs to overcome several hurdles before its clinical adaptation. However, we expect that this drug-expressing system designed by our group will lead to novel drug development instantly, because a significant amount of time, money, and labor are required for the production and purification of new protein drugs, and to the testing of the effectiveness of these agents using existing drug-development systems. In the case of minicircles encoding novel drug candidates, the effectiveness of several drug candidates can be tested via a simple DNA manipulation. If the selected drug candidates are produced using this method, several of the steps that are needed to produce the protein drugs using the current approaches can be skipped.

In conclusion, we propose a novel strategy; namely, the production of biological synthetic drugs in vitro without actual drug injection. We combined the merits of minicircles with those of biologics. There are numerous reports of the expression of proteins, such as various cytokines and intrinsic proteins, via hydrodynamic gene delivery using minicircles. However, to our knowledge, this type of attempt has never been used to express extrinsic and synthetic proteins, such as commercial drugs, in vivo. The delivery of a vector and in vivo production of a biological drug in patients may replace the pharmaceutical production process and facilitate the emergence of an innovative treatment strategy using biologics.

Methods
Production of minicircles. The minicircles mc_mock, mc_anti-IL-6R-HC, mc_anti-IL-6R-LC, and mc_sTNFR2-Fc were produced as described by Kay et al.24. Briefly, E. coli ZYC10PSST cells transformed with the parental plasmids were grown overnight at 37 °C in Terrific Broth containing 50 μg/ml of kanamycin. The cultures were combined with LB broth containing 0.02% L-(+)-arabinose and incubated at 30 °C for 5 h. Minicircle DNA was isolated using the Nucleobond Xtra Midi kit (Macherey-Nagel, Düren, Germany).

Cell culture and minicircle transfection. Human embryonic kidney (HEK293T) cells were maintained in Dulbecco Modified Eagle Medium (DMEM; Gibco Life Technology, Gaithersburg, MD) supplemented with 7.5% FBS (Gibco), 100 μM of penicillin, and 100 μg/ml of streptomycin (Gibco). HEK293T cells were transfected with minicircle vectors using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Both mc_anti-IL-6R-LC and mc_anti-IL-6R-HC were transfected into HEK293T cells to generate anti-IL-6R antibody-expressing cells. The expression of green fluorescence protein (GFP) in the cells was assessed by fluorescence microscopy at 24 h post-transfection. RA-FLS were
maintained in DMEM supplemented with 10% FBS (Gibco), 100 U/mL of penicillin, and 100 µg/mL of streptomycin (Gibco).

**Cell proliferation assay.** HEK293T cells were transfected with mc_mock, mc_sTNFR-Fc, or mc_anti-IL-6R and the conditioned media were collected 24 h post-transfection. RA-FLS were incubated with or without human IL-6 (100 ng/mL; R&D systems, Minneapolis, MN), sIL-6R (100 ng/mL; PeproTech, Rocky Hill, NJ), and TNFα (100 ng/mL) for 72 h. During the incubation, RA-FLS were treated with the conditioned media of HEK293T cells transfected with mc_mock, mc_sTNFR-Fc, mc_anti-IL-6R or PBS (as a negative control). Cell proliferation was assessed using the cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Rockville, MD), according to the manufacturer’s instructions.

**Scratch assay.** The conditioned media of HEK293T cells transfected with or without minicircles were collected 24 h post-transfection. RA-FLS were seeded onto 6-well plates (Corning-Coastar, Tokyo, Japan). When RA-FLS reached 100% confluency, the monolayer was scratched with a sterile 200 µm cell scraper. Cells were cultured with 1.5 mL of fresh DMEM supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin for 5–6 weeks of age; OrientBio, Seongnam, Korea) were added to the media, except for that of negative control cells. RA-FLS were also treated with the conditioned media of HEK293T cells. To examine whether minicircle-derived drugs affect the migration of RA-FLS in a dose-dependent manner, 250 µL or 500 µL of the conditioned media of HEK293T cells transfected with mc_mock, mc_sTNFR-Fc, or mc_anti-IL-6R were grown on the confluent media of HEK293T cells in all cases, adding the conditioned media of non-transfected HEK293T cells as required. Phase-contrast microscopy images were acquired at 0 h and 18 h after wounding were created. The invaded area was calculated as follows: percentage of invaded area = ((wounded area at 18 h)/(wounded area at 0 h)) × 100.

**Induction of arthritis and evaluation of disease severity.** Bovine type II collagen (CIIC; Chondrex, Redmond, WA) was dissolved in 0.1 M acetic acid at a concentration of 2 mg/mL at 4°C overnight. CII was emulsified 1:1 with complete Freund’s adjuvant (CFA) containing 2 mg/mL of heat-killed Mycobacterium tuberculosis (Chondrex). Female DBA1/J mice (5–6 weeks of age; OrientBio, Seongnam, Korea) were injected intradermally at day 0 and 14 with 100 µg/mL of human sIL-6R (PeproTech) at room temperature (RT) for 1 h. After washing, the plate was incubated with blocking solution for 1 h at RT, followed by incubation with 100 µL of CII/CFA emulsion. The same concentration of CII emulsified with incomplete Freund’s adjuvant (IFA; Chondrex) was injected into mice as a booster immunization 21 days after the first immunization. Five mice were used for wild-type group and seven mice were used for each group of mice treated with the same volume of normal saline. The incidence and severity of arthritis were monitored and scored as described previously. The incidence was calculated as the percentage of mice of which one or more paws were swollen among the group.

**Ethics.** All procedures involving animals were in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Guidelines and Policies for Rodent Experimentation provided by the Institutional Animal Care and Use Committee (IACUC) of the school of medicine of the Catholic University of Korea. This study protocol was approved by the institutional review board of the Catholic University of Korea (CUMC-2011-0010-03)).

**Enzyme-linked immunosorbent assay (ELISA).** To analyze the amounts of anti-IL-6R antibody and sTNFR2-Fc protein expressed by the transfected cells, the culture media of HEK293T cells transfected with the indicated minicircle vectors were analyzed by ELISA at 24 h post-transfection. The levels of sTNFR2-Fc were quantified using human sTNF-R (80 kDa) Platinum ELISA (eBioscience, San Diego, CA), according to the manufacturer’s instructions. Sandwich ELISA was performed to determine the levels of anti-IL-6R antibodies. Briefly, a 96-well microtiter plate (Corning-Coastar) was coated with commercial anti-human IL-6R antibody (B-R6; OrientalBio, Seoul, Korea) and 100 µL of the conditioned media of HEK293T cells were collected from mice. The levels of anti-IL-6R antibodies and sTNFR2-Fc in serum were analyzed by ELISA.

**Histological evaluation of arthritis.** A hind limb of each mouse was removed and fixed in 10% formalin. After decalcification in 10% (w/v) EDTA, the samples were embedded in paraffin. The 4-µm-thick sections were stained with hematoxylin and eosin (H&E), safranin O, or toluidine blue. The inflammation score and joint-destruction score were measured using the procedure of Huckel et al. Briefly, H&E-stained slides were graded blindly by three independent observers regarding the existence of synovial hyperplasia and inflammation of leukocytes; the sum of both values was used as an inflammation score. The joint-destruction score represents the sum of the score of pannus formation and that of erosion of cartilage.

**Immunohistochemical staining.** Sections (4 µm) of mouse hind paw joints were dewaxed and rehydrated, followed by heat-mediated antigen retrieval via microwaving and incubation with 3% hydrogen peroxide in methanol for 15 min. After washing with PBS, the sections were incubated for 1 h with an anti-GEF antibody (ab290; Abcam, Cambridge, MA) or rabbit IgG. After washing, the sections were incubated with a peroxidase-conjugated AffiniPure Goat anti-Rabbit IgG antibody (111-035-003; Jackson ImmunoResearch, West Grove, PA). After washing, two drops of R.T.U. Vectastain Elite ABC reagent were added, and the sections were incubated for 2 min. After rinses, the sections were reacted with the DAB (3,3'-diaminobenzidine) peroxidase substrate kit (Vector Laboratories, Burlingame, CA). Mayer’s hematoxylin was used as the counterstain. Frozen sections of mice from other experiments were also used for immunohistochemistry, to determine the localization of GFP in the tissues.

**Statistical analysis.** Statistical analysis was performed using Student’s t test. P < 0.05 was considered significant.

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**Study of the stability of biologics.** Minicircles were injected into DBA1/J mice (five animals per group) as described above. At 5, 10, and 15 days after the injection of mc_mock, mice were sacrificed and their livers were removed. The livers were analyzed by immunohistochemical staining with an anti-GFP antibody. At 5, 15, and 30 days after the injection of mc_anti-IL-6R, 100 µL of venous blood was collected from mice. At 5, 10, and 15 days after the injection of mc_sTNFR-Fc, serum was collected from mice. The levels of anti-IL-6R antibodies and sTNFR2-Fc in serum were analyzed by ELISA.
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**Author contributions**

H.Y. and Y.K. designed and performed the study, analyzed the data, and wrote the manuscript. J.K. performed cell culture. Y.A.R. produced and purified minicircle vectors. H.J. obtained tissues and stained them. S.M.J. and S.H.P. analyzed the data. J.H.J. designed and supervised the study, analyzed the data, and wrote the manuscript.

**Additional information**

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