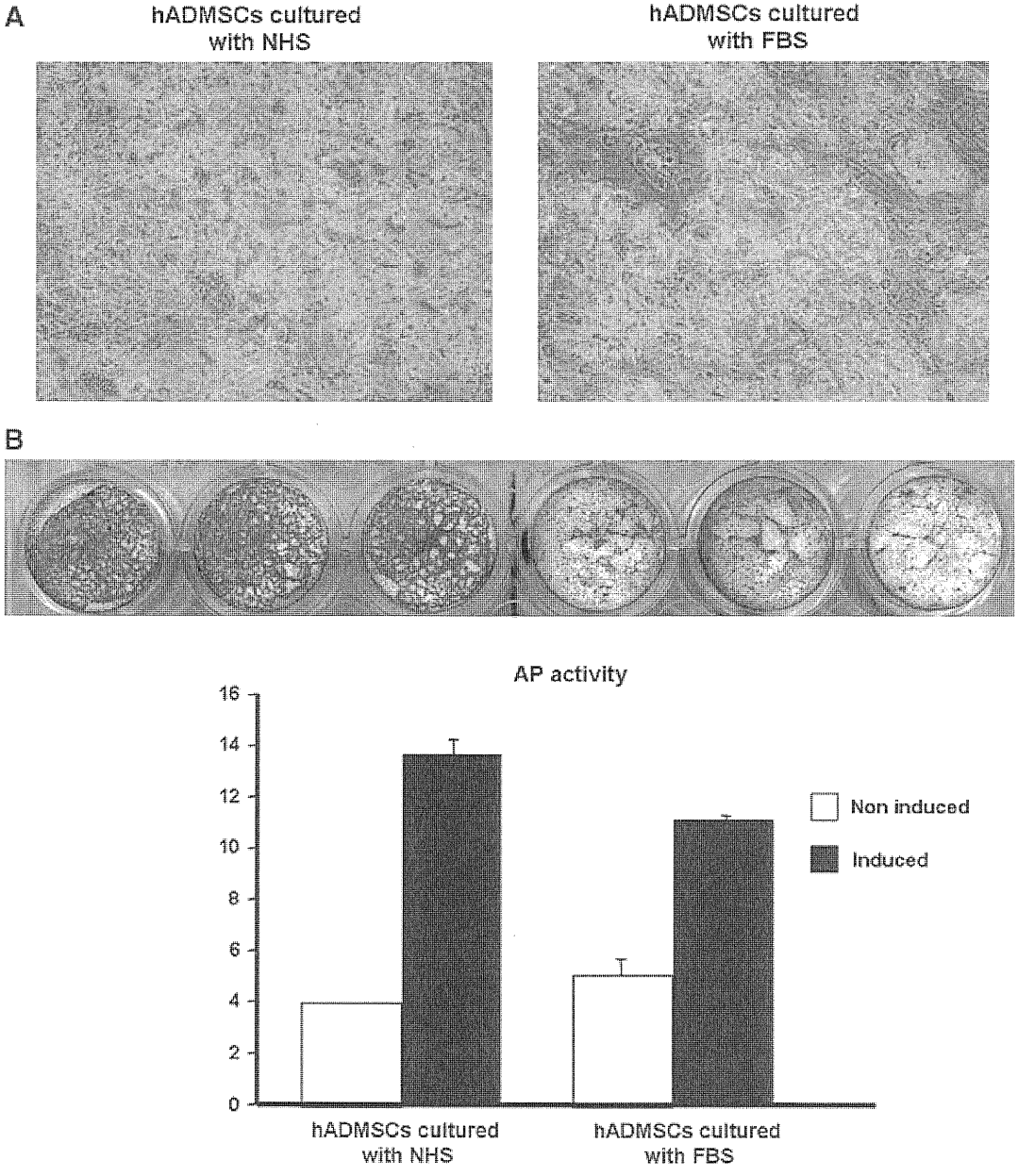

FIG. 7. Adipogenic and osteogenic differentiation potentials of hADSCs/MSCs cultured with FBS and NHS. (**A**) The efficiency of adipogenesis of hADSCs/MSCs cultured with NHS was similar to that of hADSCs/MSCs cultured with FBS. (**B**) The efficacy of osteogenic differentiation and alkaline phosphatase activity was similar between cultures with NHS and FBS in response to osteogenic induction. Data are representative of four independent experiments.

Discussion

Previous studies have reported that hESCs and BM-derived hMSCs are capable of efficient Neu5Gc uptake from culture media components.[13,14] Human serum contains high titers of natural preformed antibodies against Neu5Gc xenoantigen[20–22] and binding of these natural preformed antibodies may lead to immune responses. Importantly, this may be reflected in the published results of human clinical trials using BM-derived hMSCs cultured with FBS.[8–12] Further, in human clinical trials with FBS-grown hMSCs, antibodies against FBS have been detected.[12] However, these immune responses against human stem cells mediated by natural preformed antibodies remain in controversary.[13,23] In this study, because of the usefulness of hADSCs/MSCs as an alternative source of stem cells, we assessed the presence of Neu5Gc in hADSCs/MSCs cultured with FBS and the human immune response mediated by Neu5Gc xenoantigen.

Our study using a chicken anti-Neu5Gc polyclonal antibody showed that most of the hADSCs/MSCs cultured with FBS expressed Neu5Gc xenoantigen. This result is similar to the previous study that hESCs and BM-derived hMSCs express Neu5Gc.[13,14] In addition, our data suggested

that human natural preformed antibodies could bind to hADSCs/MSCs after exposure to fresh NHS. The subtype of natural preformed antibodies was mainly IgG, not IgM. This human IgG binding was related to the amount of Neu5Gc on the hADSCs/MSCs, because hADSCs/MSCs cultured with heat-inactivated NHS which expressed negligible levels of Neu5Gc showed negligible levels of IgG binding when exposed to fresh NHS. This result is also consistent with the previous study that anti-Neu5Gc antibodies constitute the majority of natural preformed xenoreactive antibodies besides anti-galactose-alpha 1,3-galactose (Gal) antibodies, particularly in the IgG subclass.[20,29] In effect, hADSCs/MSCs cultured with FBS may seem like xenogeneic cells to the human immune systems.

When xenogeneic grafts are transplanted into humans, binding of natural preformed antibodies that recognize xenoantigens, including Gal and Neu5Gc, mediates two types of rejection response, hyperacute rejection (HAR) and acute humoral xenograft rejection (AHXR).[30] HAR begins with binding of natural preformed antibodies to the xenogeneic epitopes on donor endothelial cells, including Gal and Neu5Gc xenoantigens, leading to complement activation by mainly classical pathway.[30] The graft is rejected within minutes to hours. Therefore, we analyzed the CMC of hADSCs/MSCs cultured with FBS that express Neu5Gc xenoantigen, using fresh NHS. However, we could not confirm the existence of CMC. The deposition of C4 and C3 fragments on hADSCs/MSCs after a short incubation with fresh NHS could not also be detected. In this issue, there are no reports describing the CMC of hADSCs/MSCs cultured with FBS that express Neu5Gc xenoantigen. Martin *et al.* reported that binding of natural preformed antibodies to Neu5Gc on hESCs mediated complement activation leading to cell death.[13] In contrast, Cerdan *et al.* reported that complement activation by anti-Neu5Gc antibody does not mediate killing of hESCs.[23] Several reasons for this discrepancy have been supposed. One is the difference of procedures used for testing cell cytotoxicity. Previous two reports detected cell cytotoxicity by propidium iodide or 7-AAD exclusion using flow cytometry. Single-cell suspension required for this procedure may cause extensive cell death even under controlled conditions. We detected cell cytotoxicity by conventional LDH release assay, which is often used in cytotoxicity assays.[32,33] The other and more possible reason is the biological difference among the human stem cells, including hESCs and hMSCs. We assessed the expression of complement regulatory proteins such as CD46, CD55, and CD59 on hADSCs/MSCs. hADSCs/MSCs were weakly positive for both CD46 and CD55 and highly positive for CD59. It is reported that HAR could be prevented by inhibiting complement activation, using transgenic animals bearing transgenes encoding human complement regulatory proteins.[34–36] Thus, it is supported that hADSCs/MSCs express complement regulatory proteins and may be largely resistant to killing by CMC mechanism. However, the expression of complement regulatory proteins on other human stem cells such as hESCs remains uncertain and further investigation is needed.

AHXR occurs when HAR is prevented, and it can be induced by low levels of natural preformed antibodies.[30] The binding of natural preformed antibodies to xenogeneic endothelial cells results in ADCC by natural killer cells, macrophages, and neutrophils, endothelial cell activation, thrombosis, and vasoconstriction.[30] It is reported that AHXR could be mediated by natural preformed antibodies against non-Gal xenoantigen,[37,38] particularly Neu5Gc xenoantigen.[39] Therefore, we analyzed the ADCC of hADSCs/MSCs cultured with FBS that express Neu5Gc xenoantigen. Our data indicated the clear existence of ADCC of hADSCs/MSCs cultured with FBS. This ADCC is supposed to be mediated by preformed natural antibodies that recognize Neu5Gc because ADCC of hADSCs/MSCs cultured with heat-inactivated NHS which expressed negligible levels of Neu5Gc could not be detected. We also analyzed the antibody-mediated cell phagocytosis of hADSCs/MSCs cultured with FBS by monocyte-derived macrophage because macrophages can target opsonized cells. However, in our study, a low level of phagocytic activity of hADSCs/MSCs cultured with FBS even in the absence of NHS was detected and this phagocytic activity clearly increased in the presence of NHS. Ide *et al.* reported that human macrophages could phagocytose porcine cells in an antibody- and complement-independent manner and elimination of Gal on porcine cells that expressed Neu5Gc did not prevent this phagocytic activity.[40] Our data indicated that hADSCs/MSCs cultured with heat-inactivated NHS which expressed negligible levels of Neu5Gc were resistant to phagocytosis mediated by human macrophages in the presence or absence of fresh NHS. Accordingly, human macrophages may be able to recognize Neu5Gc xenoantigen and phagocytose hADSCs/MSCs.

We showed here that hADSCs/MSCs cultured with FBS expressed Neu5Gc xenoantigen and that binding of natural preformed antibodies led to immune response. Based on current data, it is clear that hADSCs/MSCs should be chased without animal materials. Yamaguchi *et al.* have tried xeno-free techniques on hematopoietic stem cells by growing them on human stromal cells and using medium containing NHS.[41] To eliminate Neu5Gc on hADSCs/MSCs, we cultured them in a medium in which FBS was replaced by heat-inactivated NHS for a week after culturing with FBS. The expression of Neu5Gc on these hADSCs/MSCs was extremely reduced. Heiskanen *et al.* described that BM-derived hMSCs became decontaminated after 2 weeks of culture in a medium in which FBS was replaced by NHS, but complete decontamination was difficult to achieve by changing culture conditions.[14] Therefore, hADSCs/MSCs may not be completely decontaminated with Neu5Gc by culturing with heat-inactivated NHS for a week. However, our data suggested that human immune responses mediated by Neu5Gc on hADSCs/MSCs, such as ADCC and phagocytosis, were nearly completely prevented by this culture condition. Adipogenic and osteogenic differentiation potentials of hADSCs/MSCs cultured with heat-inactivated NHS were not less than that of those cultured with FBS. This work implies that the culture conditions avoiding renewed exposure to animal materials can reduce the expression of Neu5Gc on hADSCs/MSCs and consequently prevent human immune responses against hADSCs/MSCs. Although major complications have not been reported in the clinical trials with hMSCs cultured with FBS, human immune responses mediated by Neu5Gc may potentially influence the survival and efficacy of the transplanted cells and thus bias the published results. For clinical application of stem cell therapies based on hADSCs/MSCs, hADSCs/MSCs that presented Neu5Gc on their cell surfaces after

exposure to FBS should be cleaned up by chasing without Neu5Gc condition and thus might be rescued from xenogeneic rejection.

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AU6 ▶

Disclosure Statement

No competing financial interests exist.

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The use of leukemia inhibitory factor immobilized on virus-derived polyhedra to support the proliferation of mouse embryonic and induced pluripotent stem cells

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ABSTRACT

Human leukemia inhibitory factor (LIF) was immobilized into insect virus-derived microcrystals (polyhedra) to generate LIF polyhedra (LIF-PH) that can slowly release LIF into embryonic stem (ES) cell culture media and thus maintain ES cells in an undifferentiated state. Assays of the biological activities of LIF-PH indicated that a single addition of LIF-PH to the ES cell culture medium can support the proliferation of mouse ES and induced pluripotent stem (iPS) cells continuously for 14 days, and suggest that LIF-PH can be successfully used in the place of a periodic addition of recombinant LIF to the media every 2–3 days. The release of LIF protein from LIF-PH was determined by enzyme-linked immunosorbent assay (ELISA). Maintenance of undifferentiated state of mouse ES and iPS cells cultured with LIF-PH was determined by the detection of pluripotency-related biomarkers Oct3/4 and stage-specific embryonic antigen-1 (SSEA-1) through immunostaining and measurement of alkaline phosphatase activity. In this paper, we propose a closed culture system for mass production of ES and iPS cells that utilize a slow-releasing agent of LIF.

1. Introduction

Leukemia inhibitory factor (LIF) initially was identified as a cytokine capable of inducing the differentiation of M1 myeloid leukemia cells, and later was shown to have a strong differentiation-inhibiting activity on embryonic stem (ES) cells [1,2]. At present, mouse ES cells are cultured in an undifferentiated state on gelatin-coated polystyrene plates in the presence of LIF [3,4]. LIF belongs to the family of interleukin-6 (IL-6)-type cytokines. This family of cytokines stimulates target cells through the gp130 receptor, which is expressed in all cells of the body, while the specific cytokine receptor subunits are expressed in a cell-specific manner and induce a specific cellular response when triggered by a particular member of the cytokine family. LIF induces heterodimerization of the gp130 and LIF receptor (LIFR) upon binding to receptor subunits, and this dimerization results in the activation of associated Jak tyrosine kinases and initiation of intracellular signaling cascades [5]. Jak-mediated phosphorylation of specific tyrosine residues in the gp130 cytoplasmic domain creates docking sites for Src homology 2-containing signal transducer and activator of transcription (STAT) proteins, which are then phosphorylated and translocate to the nucleus as dimers to induce expression of specific sets of genes [6,7]. The STAT protein family has seven members, of which STAT3 is the major mediator of gp130 signals [8]. STAT3 is thought to be involved in the induction of a set of genes related to maintenance of pluripotency and self-renewal in ES cells [9,10]. Previous reports indicate that mouse LIF binding activity is specific to mouse LIFR, while human LIF can bind LIFR from either human or mouse. In addition, the binding activity of human LIF to mouse LIFR is higher than that of mouse LIF [11,12].

The disruption or removal of LIF signaling (e.g. by cytokine starvation) will trigger a signal within stem cells to differentiate [13]. Presumably, continuous activation of LIF-mediated signaling by a slow, steady release of LIF in cell culture, rather than spikes in activation and rapid withdrawal of LIF-mediated signaling observed

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during periodic addition of recombinant LIF would be beneficial for maintaining stem cell pluripotency during self-renewal. For this purpose, we have developed a new slow-releasing agent comprised of a viral protein crystalline matrix containing immobilized human LIF molecules.

Cypovirus (CPV) infections of insect larvae are characterized by the production of massive amounts of a major virus-encoded protein (polyhedrin). The polyhedrin protein molecules crystallize in the cell cytoplasm to form cubic inclusion bodies (polyhedra) that incorporate numerous virus particles. CPVs belong to a genus within the *Reoviridae* family [14]. Polyhedra function to facilitate the survival of the incorporated virus in hostile environments, stabilizing the virions and allowing long-term viability. These polyhedra are also highly resistant to both nonionic and ionic detergents and are soluble at neutral pH [15]. Previously, we have produced recombinant polyhedra derived from *Bombyx mori* cypovirus (BmCPV) without incorporation of virus particles. We developed two targeting strategies for the incorporation of foreign proteins into polyhedra. Diverse foreign proteins can be immobilized into polyhedra by fusing a polyhedron-targeting tag sequence at the C- or N-terminus of the foreign protein [16].

As described in this report, we have generated two types of polyhedra by immobilizing human LIF fused with a polyhedron-targeting tag sequence at either the C- or N-terminus. The biological effects of these polyhedra on cultured stem cells were determined and compared to those effects seen when using recombinant human LIF (rhLIF) in analogous experiments.

2. Materials and methods

2.1. Generation of LIF polyhedra

Recombinant LIF baculovirus transfer vectors were generated using GATEWAY® cloning technology (Invitrogen). The LIF gene fused to DNA encoding either the VP3 or the H1 immobilization signal was amplified by polymerase chain reaction (PCR) using primer sets containing *att*B1 and *att*B2 sequences. The forward primer 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAAGGTCTTGGCGGCAGGAG-3′ and reverse primer 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTAGAAGGCCTGGGCCAACACGGCG-3′ were used to amplify the LIF gene coupled to DNA encoding the VP3 immobilization signal, and the forward primer 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAAAGGTCTTGGCGGCAGGAGTTG-3′ and reverse primer 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTGCTAGAAGGCCTGGGCCAACACGGCG-3′ were used to amplify the LIF gene fused to DNA encoding the H1 immobilization signal. The resulting *att*B-flanked PCR products were cloned into a donor vector (pDONR221) by BP reactions of GATEWAY® system. To produce transfer vectors encoding H1 or VP3 immobilization signals, the open reading frame cloned between the *att*L1 and *att*L2 sites in the entry vectors was transferred to destination vectors (pDEST-C-VP3 or pDEST-N-H1) via LR Clonase™ reactions. The resulting transfer vectors were co-transfected into *Spodoptera frugiperda* IPLB-Sf21-AE (Sf21) cells with linearized Baculogold Baculovirus DNA (BD Pharmingen), and replication-competent baculovirus was rescued by recombination between transfer vector and the linearized viral DNA and ultimately harvested from the supernatant. Immobilization of LIF using VP3 or H1 signals was accomplished by dual infection of the recombinant baculoviruses that express the LIF fusion proteins and a recombinant baculovirus AcCP-H expressing BmCPV polyhedrin. The resulting polyhedra were recovered and purified from Sf21 cells, and detected by western blotting as previous reported in Reference [16]. Polyhedra were stored in distilled water containing penicillin (100 U/mL) and streptomycin (100 μg/mL).

2.2. Cell culture

The mouse ES cell line, EB5, was derived from *E14tg2a* (ATCC) by inserting the *Aspergillus terreus* blasticidin S deaminase gene and was provided by Dr. Niwa (Riken CDB, Kobe), and maintained as previously described in References [3,4]. In brief, EB5 cells were maintained on 6-well plates (Asahi Glass) coated with 0.1% type A gelatin (Sigma–Aldrich) in undifferentiation medium ["glow minimum essential medium" (GMEM; Invitrogen) supplemented with 1% fetal bovine serum (FBS; CELLect, MP Bioscience), 10% KnockOut Serum Replacement (KSR; Invitrogen), 0.1 mM nonessential amino acids (NEAA; Invitrogen), 1 mM sodium pyruvate (Invitrogen), 0.1 mM 2-mercaptoethanol, blasticidin (500 ng/mL; Invitrogen), and 2000 U/mL (10 ng/mL) rhLIF (Chemicon International LIF1005)]. Cultivation of undifferentiated EB5 cells harboring a blasticidin resistant gene that is fused to the *Oct-4* promoter region was achieved through blasticidin selection. EB5 passaging was accomplished by washing cells once with phosphate-buffered saline (PBS) and then incubating with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) at 37 °C for 5 min. Cell clumps were dissociated completely by pipetting in undifferentiation medium. Ten thousand cells were seeded in a 6-well plate and cultured in 2 mL of undifferentiation medium for 3 days before the next passage.

To generate mouse iPS cells, neural stem cells (NSCs) isolated from Oct-4/green fluorescent protein (GFP) transgenic mice [17] were used as cell source for reprogramming. One hundred thousand NSCs were transduced with retroviruses bearing *c-Myc*, *Klf4*, *Oct3/4* and *Sox2* genes as described in Reference [18]. Twenty-four hours post-transduction, cells were transferred to ES medium and maintained in feeder-free gelatin-coated plates. Oct-4-GFP positive cells emerged within 1 week and iPS cells (IWF-1; named after iPSc Without Feeder cell) were established in 3 weeks. Silencing of the exogenous factor and reactivation of the series of endogenous pluripotency markers were confirmed by reverse transcription (RT)-PCR. Generation of chimeric mice was confirmed following blastocyst injection.

2.3. Cell proliferation assay

For both short-term (7 days) and for long-term culture (14 days) culture, 2×10^5 cubes or 5×10^5 cubes of polyhedra containing either H1-tagged LIF (H1/LIF polyhedra) or VP3-tagged LIF (LIF/VP3 polyhedra) were mixed in 0.1% gelatin solution, coated on a polystyrene plate, and air-dried at room temperature. Cells were harvested and counted at indicated days.

2.4. Immunostaining of ES cell undifferentiation markers

Mouse ES or iPS cells were cultured with either 2×10^5 cubes of H1/LIF polyhedra, LIF/VP3 polyhedra, empty polyhedra (CP-H polyhedra), 10 ng/mL of rhLIF, or without rhLIF in the absence of blasticidin for 3 days. Cells were then fixed in 4% paraformaldehyde in permeabilization solution (0.2% Triton X-100 in PBS) for 20 min at 4 °C, rinsed with PBS, and then blocked in a blocking buffer (PBS containing 0.1% bovine serum albumin (BSA)) for 15 min at room temperature. The ES cells were incubated with a 1:100 dilution of primary antibody against Oct-4 (Santa Cruz Biotechnology, catalog #SC-5279) or against stage-specific embryonic antigen-1 (SSEA-1; Santa Cruz Biotechnology, catalog #SC-21702), and mouse iPS cells were incubated with primary antibody against SSEA-1 at 4 °C overnight followed by incubation with Alexa fluorophores (Invitrogen) for 1 h at 37 °C. Alkaline phosphatase (ALP) activity of cultured cells was detected with a Vector Blue® Alkaline Phosphatase Subtraction Kit (Vector Laboratories) according to the manufacturer's instructions. Cells were observed by either fluorescence microscopy or by phase-contrast microscopy using an Olympus CKX-31 inverted microscope.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Concentration of LIF in culture media was determined by ELISA. Two hundred thousand cubes of CP-H polyhedra or either LIF/VP3 or H1/LIF polyhedra were added in undifferentiation medium to 1×10^5 EB5 cells. The release of LIF from polyhedra in fresh undifferentiation medium was measured at days 0 (1 h after adding polyhedra to the cell culture), 1, 2, 3, 4 or 5, and was determined using a Human LIF Quantikine ELISA kit (R&D Systems) in accordance with the manufacturer's protocol. The release of LIF from polyhedra in a conditioned medium harvested from culture medium used to keep EB5 in confluence for 24 h was also determined with this kit. The amount of soluble LIF was measured by absorbance at 450 nm with a microplate reader (Model 680, Bio-Rad).

Activation of STAT3 was also determined by ELISA. Ten thousand EB5 cells were cultured without rhLIF in the presence of blasticidin for 3 days to generate a LIF starvation culture, and then either 2×10^6 cubes of H1/LIF polyhedra or 10 ng/mL rhLIF was added to the cell culture. The cells were harvested at designated time points, mixed with ice-cold cell lysis buffer (Cell Lysis Buffer (10×); Cell Signaling) with 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma) and incubated at 4 °C for 5 min after assessing the cell number. The cell lysate was centrifuged at 14000×*g* for 10 min at 4 °C, and the supernatant was then collected. Soluble phospho-STAT3 (Y705) levels in the supernatant were measured by ELISA using a PathScan® plate in accordance with the manufacturer's protocol. Horse radish peroxidase (HRP)-conjugated antibody was used for the detection of phospho-STAT3, and absorbance at 450 nm was measured with a microplate reader (Model 680, Bio-Rad).

2.6. Western blotting

Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot detection was performed by suspending 4×10^5 cubes of each type of polyhedra in 30 μL of SDS-PAGE sample buffer (50 mM Tris–HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol), followed by boiling for 5 min, cooling on ice, and separating the polyhedra lysates by SDS-PAGE at 25 mA. Duplicate lanes for each polyhedra sample were prepared, and following electrophoresis one-half of the gel was transferred onto a nitrocellulose membrane at 54 mA for 90 min for western blotting while the other half was stained with coomassie blue. The blotted membrane was incubated with an anti-human LIF monoclonal primary antibody (R&D systems) diluted 1:250 in 3% skim milk/Tris-buffered

saline with Tween-20 (TBS-T) for 2 h at 25 °C, followed by a second incubation at 25 °C for 2 h with an HRP-conjugated anti-mouse secondary antibody (Bio-Rad) diluted 1:3000 in 3% skim milk/TBS-T. After several washes, the membrane was developed with an immunostaining kit (HRP-1000; Konica) according to the manufacturer's instructions.

3. Results

3.1. Immobilization of human LIF into BmCPV polyhedra

The viral protein VP3 mediates incorporation of BmCPV into polyhedra, and a stretch of 52 amino acids within this protein is sufficient to direct immobilization of foreign proteins into polyhedra. This 52-aa sequence was fused to the C-terminus of LIF to direct LIF uptake into polyhedra, and is termed VP3-tag. Similarly, a stretch of 30 amino acids from the N-terminal alpha helix of the BmCPV polyhedrin protein can be used to direct immobilization of foreign proteins into the cubic polyhedra structure [15,16,19], and this 30-aa sequence (termed H1-tag) was fused to the N-terminus of LIF to direct LIF immobilization into polyhedra. Human LIF protein bearing either a VP3-tag or a H1-tag was immobilized into polyhedra to generate two types of LIF polyhedra that can slowly release LIF into the culture medium, namely H1/LIF polyhedra and LIF/VP3 polyhedra. The amount of immobilized LIF, the LIF release rate from polyhedra, and the biological activities of the released protein were examined in biological assays, with the two tagging methods yielding differing outcomes.

The schema for generation of the two types of LIF polyhedra and photos of LIF polyhedra are shown in Fig. 1A. Immobilization of LIF protein into polyhedra was confirmed by the detection of LIF protein by western blotting (Fig. 1B). LIF protein can be immobilized into polyhedra by either the H1-tag or the VP3-tag. However, the intensity of LIF bands in the western blot indicates that LIF protein was more efficiently immobilized into polyhedra when the H1-tag was used.

3.2. Release of LIF in cell culture media

Release of LIF from H1/LIF and LIF/VP3 polyhedra in culture medium was determined by ELISA. Ten thousand EB5 cells were cultured with 2×10^5 cubes of H1/LIF polyhedra, and release of LIF was observed 1 h after the culture was initiated (day 0). However, the same dose of LIF/VP3 polyhedra did not release detectable levels of LIF, with measurements showing only background-level signals, as low as empty polyhedra (CP-H polyhedra) derived from an infection of Sf21 cells with AcCP-H alone (Fig. 2). The release of LIF from H1/LIF polyhedra peaked 2 days after the culture was initiated, and lasted as long as 5 days so far measured. On the other hand, rhLIF levels begin to diminish at day 3 and are drastically reduced at day 4 days, with levels almost 1/10 of the initial dose. The rhLIF levels were barely detectable at day 6. Interestingly, the amount of rhLIF added to the cell culture is drastically reduced within 1 h. A likely explanation for this drop is that some portion of rhLIF binds to LIF receptor, while the majority of rhLIF molecules stick to the wall of culture dishes or are degraded soon after addition to the cell culture media. The release of LIF was observed within 1 h when H1/LIF polyhedra were soaked in fresh cell culture media (without cells), but not in PBS (Fig. 2). Also interestingly, the amount of LIF released was suppressed when H1/LIF polyhedra were incubated with conditioned media alone (without cells) or added to overgrown EB5 cell culture that had been cultured with mouse recombinant LIF. The ELISA kit used in these studies detects rhLIF but not mouse recombinant LIF (data not shown). These data suggest that the culture media may contain substance(s) that promotes the degradation of polyhedra, while the conditioned media may have factor(s) that antagonize or neutralize such putative digestive activities of substance(s) found in fresh culture media.

3.3. Maintenance of pluripotent stem cells with H1/LIF polyhedra

Colonies of pluripotent mouse ES or iPS cells having a dome-like morphology require a continuous supply of LIF to proliferate in the undifferentiated state, while the colonies start to differentiate and become flat or take on an epithelium-like appearance in the absence of LIF. EB5 cells cultured with rhLIF or on H1/LIF or LIF/VP3 polyhedra-coated 6-well plates are shown in Fig. 3. Notably, the cell culture with 2×10^5 cubes of H1/LIF polyhedra, as well as that with 10 ng/mL rhLIF, maintained dome-shape colonies that were positively-stained with ALP, but such findings from the culture with the same dose of LIF/VP3 polyhedra were not observed (Fig. 3). In accord with the LIF release data shown in Fig. 2, these results suggest that the level of LIF release from LIF/VP3 polyhedra was not sufficient to maintain the undifferentiated state of mouse ES or iPS cells. The maintenance of the undifferentiated stage of pluripotent stem cells was further evaluated by the detection of pluripotency-related biomarkers such as Oct3/4 or SSEA-1. Mouse ES cell colonies maintained a dome-like appearance and retained the expression of Oct3/4 and SSEA-1 molecules when the cells were cultured with 2×10^5 cubes of H1/LIF polyhedra or 10 ng/mL of rhLIF, but not with LIF/VP3 polyhedra or with CP-H polyhedra (Fig. 4A and B). An epithelium-like colony emerged and loss of SSEA-1 or Oct3/4 expression was observed when the cells were cultured in the absence of rhLIF (data not shown), as was observed in cell cultures with CP-H or LIF/VP3 polyhedra.

Like the mouse ES cell colonies, mouse Oct-4-GFP knock-in iPS cell colonies (iWF-1) cultured with rhLIF and H1/LIF maintained a dome-shape morphology, SSEA-1 expression at a molecular level, and Oct3/4 at a transcriptional level, but such cultures in the presence of LIF/VP3 or CP-H polyhedra did not (Fig. 4C and D), analogous to the results shown in Fig. 3. Morphology of the iPS cell colonies cultured with LIF/VP3 polyhedra was distinct from that with H1/LIF polyhedra. Dish-like colonies emerged when cultured with LIF/VP3, but the rim of these colonies were detached from the gelatin-coated dish and formed embryoid body-like cell clumps. These cell clusters did not express SSEA-1 at the center of the colony. Interestingly, EB5 cells spontaneously start to differentiate as an adhesive cell, while iWF-1 cells lose adhesive properties and start to differentiate following LIF depletion. It is presumed that difference in epigenetic status may account for a distinct cellular behavior in differentiation upon LIF depletion in these two cell lines. The exact mechanism shall be elucidated in future study.

3.4. Addition of H1/LIF polyhedra sustains ES cell growth in a dose-dependent manner

The effect of LIF polyhedra on mouse ES iPS cell proliferation during short-term (7-day) culture was determined by assessing cell number at days 3 and 5. Of particular note, the proliferation potential afforded by 2×10^5 cubes of H1/LIF polyhedra is equivalent to that of 10 ng/mL rhLIF and far superior to that of LIF/VP3 (Fig. 5A and C). In addition, ES cells proliferated in a dose-dependent manner within the range of 7.5×10^3 to 2×10^5 cubes of H1/LIF polyhedra (Fig. 5B).

The slow release effect of H1/LIF polyhedra was determined by assessing cell number during ES cell culture over 2 weeks. ES cells were cultured either with 2×10^5 cubes or 5×10^5 cubes of H1/LIF polyhedra or 10 ng/mL rhLIF, and cell numbers were determined at days 3, 5, 7, and 14. The rhLIF was added to the culture media at days 0, 3, 5, 7, 10 and 12 to sustain cell proliferation, while H1/LIF polyhedra were added just once, at the start of the culture. H1/LIF

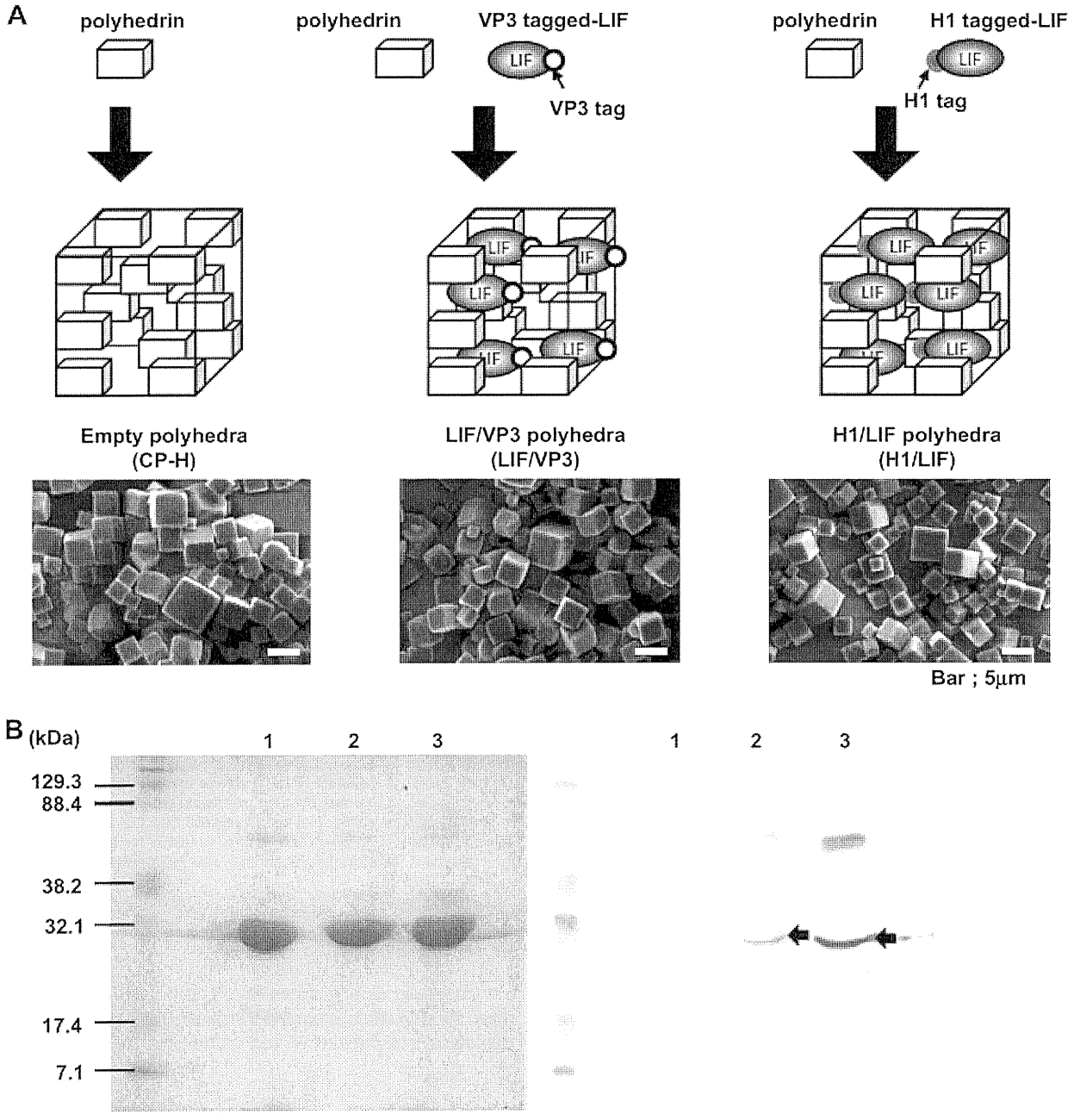

Fig. 1. (A) Schema for the generation of LIF slow-releasing agents (LIF-containing polyhedra), and corresponding photos of polyhedra crystals. Either H1-tagged LIF or VP3-tagged LIF was immobilized into polyhedra to generate two types of slow-releasing agents, namely H1/LIF polyhedra (H1/LIF) or LIF/VP3 polyhedra (LIF/VP3). Empty polyhedra (CP-H) consisting solely of BmCPV polyhedrin protein were used as a control. (B) Detection of LIF immobilized into polyhedra by SDS-PAGE and western blotting. LIF immobilized into polyhedra was analyzed by electrophoresis on 12.5% polyacrylamide gels (left panel) and detected by western blotting (right panel) with an anti-LIF monoclonal antibody. Lanes 1, 2, and 3 correspond to CP-H, LIF/VP3, and H1/LIF samples, respectively. Arrows show the bands corresponding to human LIF molecule.

polyhedra showed a slow-releasing effect, as 5×10^5 cubes of H1/LIF polyhedra sustained ES cell proliferation at a comparable level to that seen with the cell culture where 10 ng/mL rhLIF is added every 2–3 days (Fig. 5D).

3.5. Signaling delivered by H1/LIF polyhedra is persistent

Propagation of LIF-stimulated intracellular signaling is mediated by STAT3 activation [20], which can be determined by the detection of phosphorylated STAT3. Indeed, addition of rhLIF or H1/LIF polyhedra to a rhLIF-starved EB5 cell culture induced a spike in STAT3 phosphorylation at 3 and 6 h (Fig. 6). The STAT3 phosphorylation induced by rhLIF rapidly falls to basal levels by day 3 at the single cell level, and additional 10 ng/mL rhLIF is needed to resume increased STAT3 phosphorylation levels. STAT3 phosphorylation was also assayed 6 h after the addition of rhLIF on days 3 and 5. A similar induction of STAT3 phosphorylation was observed 6 h after adding rhLIF on days 3 and 5 of the culture followed by reduction of STAT3 phosphorylation at days 5 and 7. However, the magnitude of the phospho-STAT3 induction is attenuated over the course of the culture at the single cell level due to an increased total cell number (Fig. 6). In contrast, when 2×10^5 cubes of H1/LIF polyhedra were

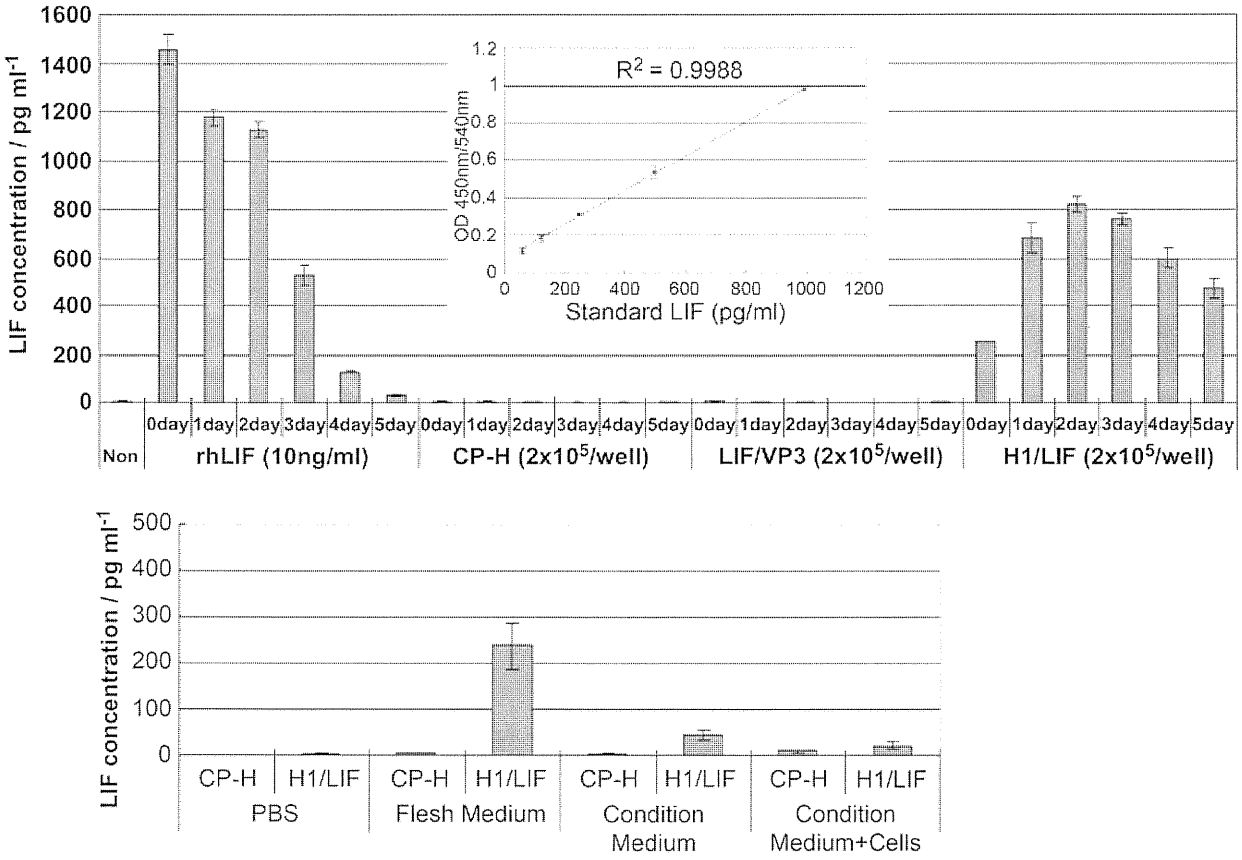

Fig. 2. Concentration of human LIF in ES cell culture media from designated culture conditions. ES cells were cultured either with 10 ng/mL rhLIF, or with 2×10^5 each of cubes of empty CP-H polyhedral (CP-H), LIF/VP3 polyhedra (LIF/VP3), or H1/LIF polyhedra (H1/LIF) (Upper panel). The amount of LIF in culture media from an individual sample was determined by ELISA and the measured value is shown as a bar, with an error bar indicating standard deviation (*X*-axis, different treatments and times; *Y*-axis, amount in pg/ml). Samples taken 1 h after the culture was initiated were designated as being collected at day 0. In addition, the amount of human LIF released from 2×10^5 cubes of CP-H or H1/LIF polyhedra that were suspended for 1 h in PBS, fresh media for EB5 (Fresh Medium), conditioned media from 3-day-old EB5 culture (Condition Medium), or 3-day-old EB5 cell culture (Condition Medium + Cells) was also determined (Lower panel). The inset shows the calibration curve and the best-fit lines used for converting optical density (OD) to concentration values.

added to EB5 cells at the start of the culture, the sharp activation of STAT3 decreases slowly over time, as determined by measurements made at day 3 + 6 h, day 5 + 6 h, and day 7 + 6 h. The concentration of LIF in culture media under various culture conditions shown in Fig. 2 also supported these results.

4. Discussion

Our study demonstrates that H1/LIF polyhedra, which immobilized human LIF into polyhedra via the H1-tag, possess long-acting biological activities in ES cell culture, with effects comparable to

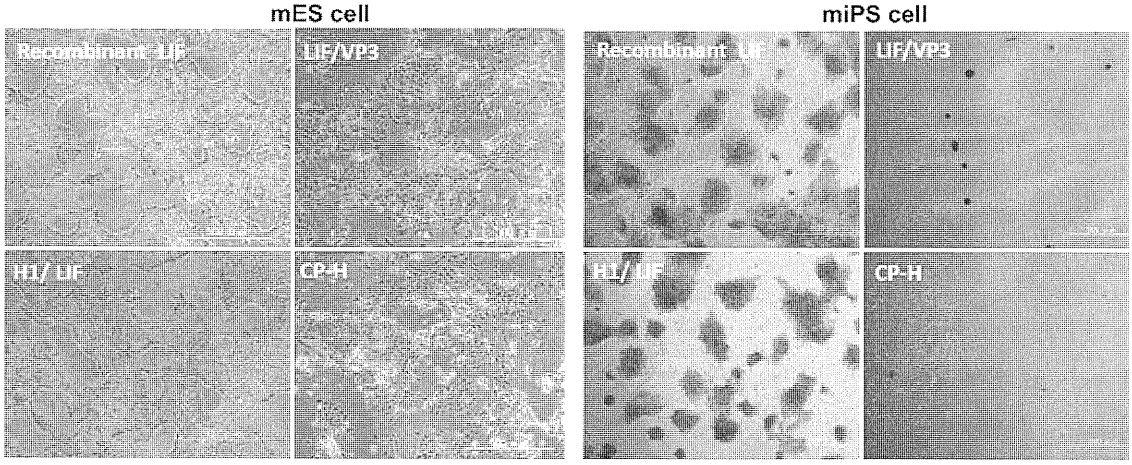

Fig. 3. Biological effect of H1/LF and LIF/VP3 on the maintenance of ES cells. Mouse ES cells (EB5 cells; left panels) or mouse iPS cells (iWF-1 cells; right panels) were cultured with 10 ng/mL rhLIF (upper left) or with 2×10^5 cubes of LIF/VP3 (upper right), H1/LIF (lower left) or CP-H (lower right) polyhedra for 5 days, followed by ALP staining.

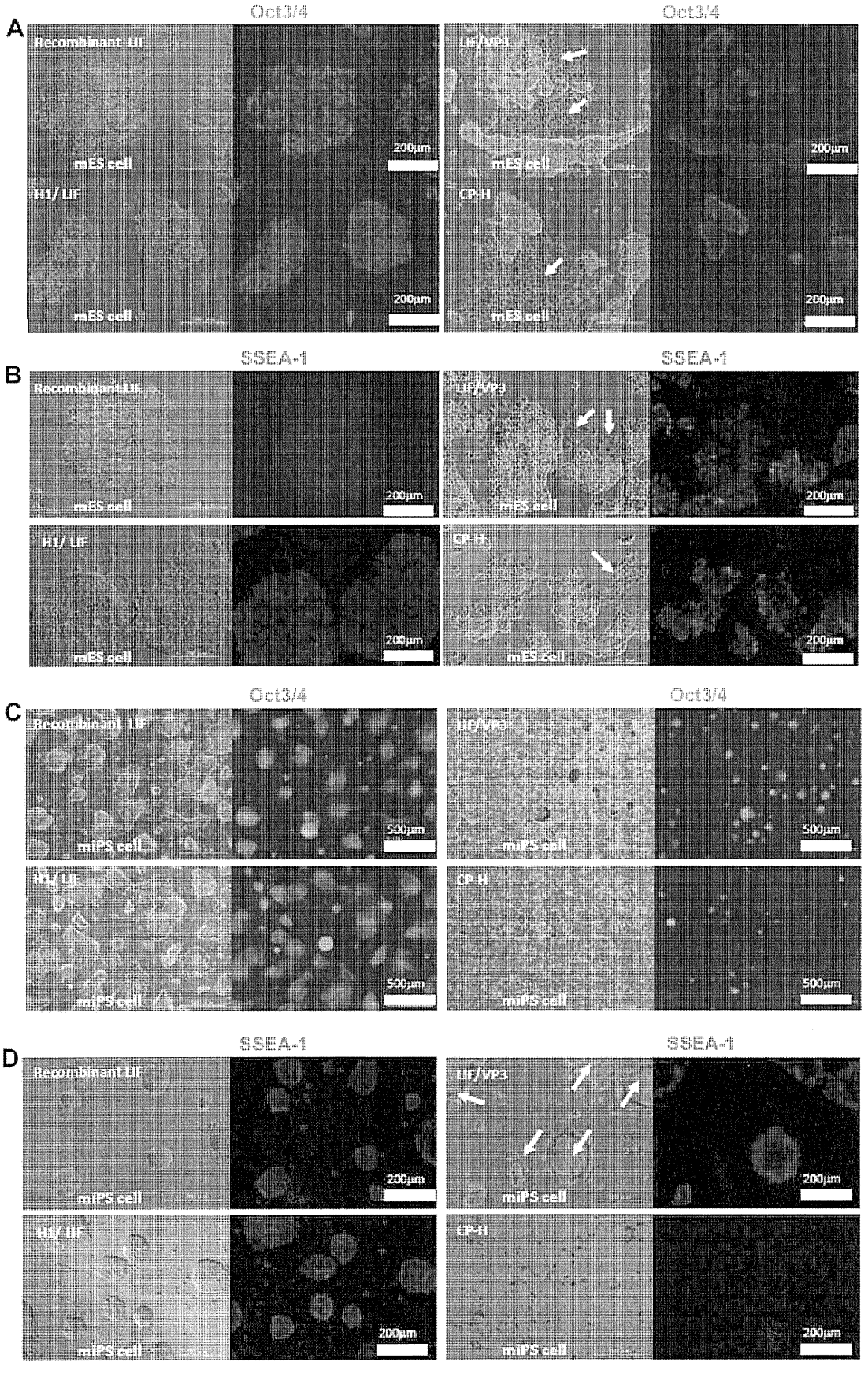

Fig. 4. Expression of pluripotency-related markers in ES cells cultured with polyhedra. Expression of Oct3/4 (A), SSEA-1 (B) in cultured EB5 cells, or Oct3/4 (C), SSEA-1 (D) in cultured mouse iPS cells (iWF-1 cells) with 10 ng/mL rhLIF (upper left) or 2×10^5 cubes of LIF/VP3 (upper right), H1/LIF (lower left) or CP-H (lower right) polyhedra are shown. Cells were cultured with respective agents for 5 days and fixed for immunostaining. A phase-contrast image (left) and the corresponding immunostaining image (right) under a fluorescent microscope are shown.

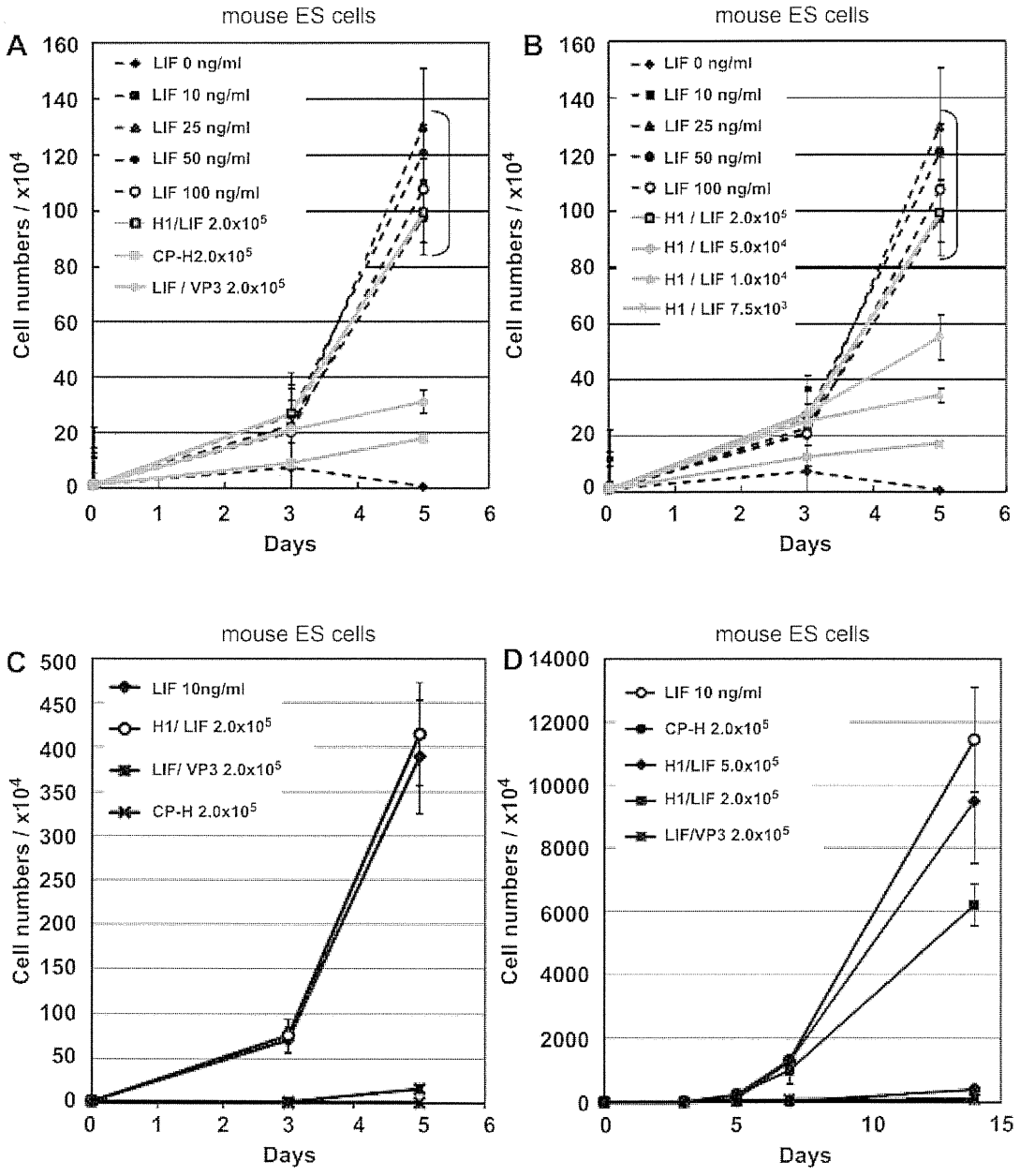

Fig. 5. Addition of H1/LIF promotes the growth of pluripotent stem cells in a dose-dependent manner. Results are shown as linear graphs with mean and standard deviation (SD; indicated by error bars) derived from 3 independent experiments. A: mouse ES cells proliferate in the presence of H1/LIF polyhedra. Either 2×10^5 cubes of H1/LIF, LIF/VP3, or CP-H polyhedra were added to 1×10^4 EB5 cells at the beginning of the culture, while 10, 25, 50 or 100 ng/mL rhLIF was added at days 0 and 3 of the culture to sustain cell proliferation. The number of cells was assessed at days 3 and 5. B: ES cells proliferate by the addition of H1/LIF polyhedra in a dose-dependent manner. Either 7.5×10^3, 1.0×10^4, 5.0×10^4 or 2.0×10^5 cubes of H1/LIF polyhedra were added to 1×10^4 EB5 cells at the beginning of the culture, while 10, 25, 50 or 100 ng/mL rhLIF was added at days 0 and 3 of the culture to sustain cell proliferation. The number of cells was assessed at days 3 and 5. C: Mouse iPS cells proliferate in the presence of H1/LIF polyhedra. Either 2×10^5 cubes of H1/LIF, LIF/VP3, or CP-H polyhedra were added to 1×10^4 EB5 cells at the beginning of the culture, and 10 ng/mL rhLIF was added at days 0 and 3 to sustain cell proliferation. The number of cells was assessed at days 3 and 5. D: H1/LIF polyhedra sustain the proliferation of mouse ES cells for 2 weeks. Ten thousand EB5 cells were cultured with either 10 ng/mL rhLIF, 2×10^5 cubes or 5×10^5 cubes of H1/LIF polyhedra, 2×10^5 cubes of LIF/VP3 polyhedra, or 2×10^5 cubes of CP-H polyhedra for 14 days. The number of cells was assessed at days 3, 5, 7, and 14. rhLIF was added at days 0, 3, 5, 7, 10 and 12, while polyhedra were only added at the start of corresponding cultures.

those seen for a periodic administration of rhLIF protein to the culture media. The mechanism underlying how LIF is released from H1/LIF polyhedra but not from LIF/VP3 polyhedra remains elusive, and further protein crystal analysis is needed to provide full answers. Nonetheless, our findings suggest that inexpensive H1/LIF polyhedra can be productively used to sustain a long-term and a closed mass culture system.

Recently, a biocarrier using immobilized LIF on a maleic anhydride copolymer thin-film was reported [21]. Here, the immobilized LIF protein regulated the pluripotency of ES cells growing on the film for 2 weeks. The ES cell colony spread radially and notably the cells in the center of the colony, not at the rim of the colony, could use up LIF immobilized in the film, resulting in differentiation from the center of colony due to deficient LIF stimulation. In this report, we demonstrate the biological potential of immobilizing LIF into polyhedra (e.g. H1/LIF), which can then be mixed with the culture medium as a soluble, slow-releasing agent of LIF, thereby obviating the need to add rhLIF to the culture medium every 2–3 days to maintain ES cell proliferation. Furthermore, unlike rhLIF that induces spikes in STAT3 phosphorylation (Fig. 6) and cellular

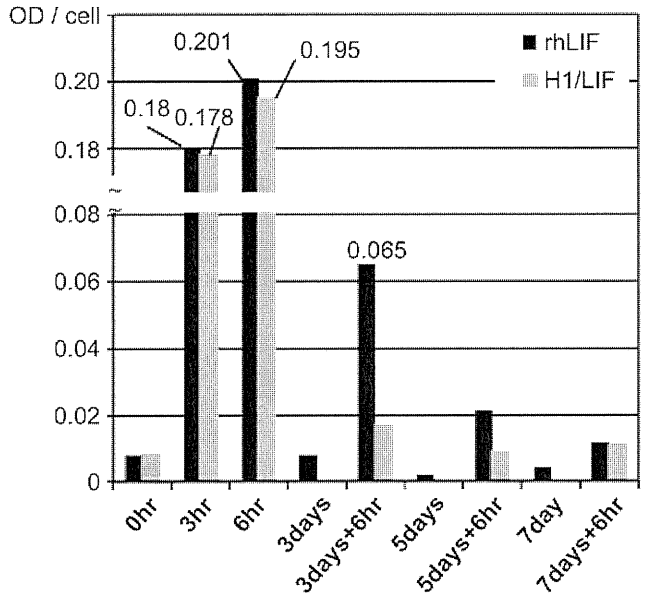

Fig. 6. STAT3 activation at a single cell level. Ten thousand EB5 cells were cultured either with 10 ng/mL rhLIF (black bar) or 2×10^5 cubes of H1/LIF polyhedra (gray bar). The activated form of STAT3 (phosphorylated STAT3), measured in OD units at designated time points at a single cell level, is shown.

response [13], use of the slow-releasing agent H1/LIF provided a persistent STAT3-mediated signal activation through continuous LIF release into the culture medium. This slow-releasing property, we presume, will be beneficial for the maintenance and proliferation of ES cells by avoiding the fluctuations (or disruptions) in intracellular signaling through periodic ligand stimulation and starvation.

Recently, two distinct pluripotency stages of the mouse ES cell, namely the inner cell mass (ICM)-type stem cell and Epiblast-type stem cells (EpiSCs) have been reported [22–27]. ICM-type mouse ES cells are "bona fide" pluripotent stem cells representing the pre-implantation blastocysts that are able to contribute to chimerism when placed back into blastocysts, and require LIF for the maintenance and proliferation of ES cells. While EpiSCs ES cells, which represent the post-implantation stage epiblasts, demonstrate the potential to differentiate into three germ lines *in vitro*, these cells are incapable of contributing to chimerism. EpiSCs require basic fibroblast growth factor (bFGF) for the maintenance and proliferation of ES cells. Both human ES cells and human iPS cells seem to correspond to the EpiSCs with respect to colony morphology, gene expression profiles, and cytokine requirement [25], but can be converted to the ICM-type stem cell stage by cultivation [28] or by constitutive activation of *Klf2/Klf4* genes [26,27] in the presence of LIF. These reports suggest the potential applications of H1/LIF polyhedra for the maintenance of ICM-type human ES cells and human iPS cells in a closed culture system in future study. In the course of reviewing process of this paper we have succeeded to culture human naïve iPS cells with H1/LIF and show the result in Supplementary figure.

5. Conclusions

Human LIF was immobilized into insect virus polyhedra (LIF polyhedra) and release of LIF protein from LIF polyhedra was observed for at least 5 days. Mouse ES cells and mouse iPS cells were proliferated by adding LIF polyhedra in a dose-dependent manner. Both ES cells and iPS cells formed colonies and were stained by Alkali phosphatase positively. Expression of Oct3/4 and SSEA-1 of ES and iPS colony was also determined. A single addition of LIF polyhedra to the ES cell culture medium supported the proliferation of both ES cells continuously for 14 days.

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Appendix

Figures with essential colour discrimination. Figs. 1, 5 and 6 in this article are difficult to interpret in black and white. The full colour images can be found in the online version, at doi:10.1016/j.biomaterials.2010.12.063.

Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2010.12.063.

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Original Article

Secreted Frizzled Related Protein 4 Reduces Fibrosis Scar Size and Ameliorates Cardiac Function After Ischemic Injury

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Expression of the Wnt modulator secreted frizzled related protein 4 (Sfrp4) is upregulated after heart ischemic injury. We show that intramuscular administration of recombinant Sfrp4 to rat heart ischemic injury and recanalization models prevents further deterioration of cardiac function after the ischemic injury. The effect of Sfrp4 persisted for at least 20 weeks when Sfrp4 was administered in a slow release system (Sfrp4-polyhedra) to both acute and subacute ischemic models. The histology of the dissected heart showed that the cardiac wall was thicker and the area of acellular scarring was smaller in Sfrp4-treated hearts than in controls. Increased amounts of both the inactive serine 9-phosphorylated form of glycogen syntase kinase (GSK)-3β and the active form of β-catenin were observed by immunohistology 3 days after lateral anterior descendant ligation in control, but not in Sfrp4-treated hearts. All together, we show that administration of Sfrp4 interferes with canonical Wnt signaling that could mediate the formation of acellular scar and consequently contributes to the prevention of aggravation of cardiac function.

Introduction

The consequences of myocardial infarction remain the leading causes of death in the developed world. Death caused by myocardial infarction can result from either acute loss of heart function immediately after the infarct or chronic heart failure after initial survival (due to either the mildness of the infarct or successful medical intervention). Heart pump function lost after ischemic injury cannot be recovered by conventional medication, and the ischemic heart has therefore been thought to be an ideal target for regenerative medicine utilizing stem cell transplantation. However, transplantation of putative tissue-derived cardiac progenitors have failed to produce any promising clinical benefits.[1–4] Cardiac progenitors derived from ES cells have been shown to provide a small improvement in cardiac function in animal ischemic models[5] and such therapies may become useful in the future. Presently, however, several critical issues (e.g., the cell purification process, low cell survival, and immune rejection) remain to be resolved before such cells can be used in the clinic.[6] These facts prompted us to search for secreted factors that underlie cardiac functional recovery and that might improve cardiac function after ischemic injury using microarray data.

We have previously made use of gene expression data from both a mouse myocardial infarction model[7] and a rat infarction and treatment model[8] to identify genes (a) that are upregulated after infarction, (b) whose expression is further increased as a result of myoblast sheet application (which provides therapeutic benefit),[9] and (c) that encode secreted factors that have the potential to act when applied exogenously to the heart. Secreted frizzled related protein 4 (Sfrp4), Midkine, and pleiotrophin all met these criteria (Supplemental Table S1, available online at www.liebertonline.com/ten). We have previously reported that application of Midkine to ischemic areas can partially block postinfarct deterioration of heart function[9]; however, of the remaining genes, only *Sfrp4* showed obvious effects in a preliminary screen; hence in this work we focused on the therapeutic potential of Sfrp4.

Sfrp proteins are characterized by a frizzled-like cysteine-rich domain (CRD) in the N-terminal half and form a family of soluble proteins (Sfrp1-5) that can be subdivided into two

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groups; Sfrp1, Sfrp2, Sfrp5, and Sfrp3, and Sfrp4 on the basis of sequence homology.[10] Frizzled proteins act as receptors for Wnt ligands and it is thought that the Sfrps may also interact with Wnt proteins.[11] The expression patterns of the Sfrps during chick, mouse, and xenopus embryonic development overlap with their Wnt counterparts,[12–14] supporting the idea that Sfrps modulate Wnt signaling through the frizzled-like CRDs.[15,16] However, it remains unclear whether Sfrps physiologically interact with Wnts through the CRD or even whether they act as Wnt antagonists or agonists.[17,18] Additionally, since many Wnt proteins can interact with several different Frizzled receptors, it is still unclear which ligand–receptor interactions are physiologically relevant and what signaling results from which interactions.

Heart ischemic injuries can result from either permanent or temporary occlusion of cardiac arteries. Although these injuries have different pathologies, both result in the death of cardiomyocytes and the replacement of cardiac tissue with scar tissue. Permanent ischemic injuries result in cell death due to the resulting anoxic conditions, whereas transient ischemia is believed to cause secondary tissue damage through the diffusion of inflammatory factors from the ischemic area (reperfusion injury). Patients who have undergone per-cutaneous trans-coronal angioplasty intervention after coronary occlusion frequently present physiological conditions characteristic of reperfusion injury. Permanent ischemic damage can be modeled using a ligation of the lateral anterior descendant (LAD) branch, whereas transient ischemia can be modeled by a temporary LAD ligation applied during the period of surgery.[19]

Both transient and permanent ischemic injuries result in cell death, scar formation, and a tissue remodeling process that continues over a period longer than 1 month.[20] It therefore seems likely that a single application of a protein product to the ischemic area will only have an effect during the early stages of tissue remodeling, and make it desirable to identify means of providing a long-term delivery of protein products to specific areas of tissues. It has previously been shown that proteins can be immobilized within insect viral particles (polyhedrons). Such particles are slowly degraded by extracellular proteases, resulting in the gradual release of the immobilized proteins.[21,22] Polyhedra particles may thus facilitate the *in vivo* long-term delivery of therapeutic proteins to injured tissues.

In this report, we demonstrate for the first time that the Wnt signal modulator Sfrp4 is upregulated in ischemic heart and that administration of Sfrp4 improves cardiac function after both permanent and transient ischemic injury. We further show that the therapeutic effect can be prolonged by the immobilization of Sfrp4 in polyhedra particles and demonstrate a number of different means by which such particles can be applied to ischemic regions. Our data suggest that the therapeutic effect of Sfrp4 application is due to a reduction in acellular scar tissue formation resulting from an inhibition of canonical Wnt signaling.

Materials and Methods

Rat heart ischemic and recanalization models

All animal experimental protocols were reviewed by the animal experiment committee of the Foundation for Biomedical Research and Innovation. Seven-week-old male Sprague-Dawley rats (220–250 g; Japan SLC) were used to generate a heart infarction model by ligating the left coronary anterior descendent branch (LAD ligation model) or a recanalization model by ligating LAD for 1 h followed by release of the ligation. Both ischemic and recanalization models were generated by open chest surgery as previously reported.[7] For the anesthesia of the rats, 2.5 mL of anesthetic solution (10% v/w Ketamine [Ketalar, Daiichi-Sankyo Propharma]) and 2.5% v/w Xylazine (Selactar, Japan Bayer Medical) in an isotonic solution in Solita T1 (Shimizu Pharmaceutical Co.) was administered to the rats intraperitoneally. A respiratory device (Respirator SN-480-7x2T; Shinano) was used to aid the ventilation during the open chest LAD operation.

Sfrp4 administration

Sfrp4 was administered to ischemic regions by three different means: (1) by intramuscular (IM) injection of soluble protein, (2) by IM injection of Sfrp4 containing polyhedra (S-PH), and (3) by combining S-PH with a biodegradable vehicle.

Soluble IM injection. Five or 20 μg recombinant human Sfrp4 (sFRP-4; R&D Systems, Inc.) in 50 μL phosphate-buffered saline (PBS) was mixed with 50 μL collagen type I gel (BD Biosciences) and injected to the ischemic border zones. Satisfactory injection of reagents with 26G needles (Terumo) was confirmed several times by injecting blue ink and observing ink staining in wall muscle after sacrifice.

S-PH preparation. The open reading frame of the human *Sfrp4* gene (CR 541755) was amplified by polymerase chain reaction (PCR) with the 5′Sfrp4 and 3′Sfrp4 primers. Human Sfrp4 protein was then translated as a fusion protein with VP3, which facilitates immobilization within polyhedra, to generate S-PH in S9 cells as described.[8,9] We estimate that the average mass of Sfrp4-VP3 immobilized in a polyhedron (one cube) is $\sim 8\times10^{-3}$ ng (the average volume of a polyhedron is $5\times5\times5\ \mu m^3$ with a density of 1.3 g/mL and ~5% of the total mass corresponds to Sfrp4-VP3).[21,22] Hence 2.5×10^6 polyhedra is roughly equivalent to 20 μg of recombinant protein. Polyhedra cubes were applied in a number of ways: (1) 2.5×10^6 cubes of polyhedra were mixed with 100 μL of PBS and injected to ischemic border zones, (2) 5×10^6 cubes of polyhedra were immobilized on a 9×9 mm collagen type I sheet by air-drying and applied to the ischemic heart just after LAD ligation, or (3) 5×10^6 cubes of S-PH were mixed with 150 μL of fibrin glue Bolheal (Teijinn Pharma) and plastered to the ischemic area directly by reopening the chest 2 weeks after LAD ligation.

Echocardiographic assessment of cardiac function

Rat left ventricular (LV) functions were echocardiographically monitored with a SONOS 7500 (Philips Electronics). Two operators performed the ultrasound measurement in a blind fashion. The LV end-systolic area, LV end-diastolic area (LVEDA), LV dimensions at end-systole, and LV dimensions at end-diastole (LVDd) were determined with the device. Ejection fraction (EF) was calculated by the modified Simpson method (disc method). The mean of measured long

axis (L) in several cycles was used for the calculation of EF. LV percent fractional shortening (% FS) was calculated by dividing the difference between the LVDd and end-systolic dimension with LVDd, and shown as percentage.

Functional area change (%) was calculated by dividing the difference between LVEDA and LV end-systolic area with LVEDA, and shown as percentage. The statistical significance of treatment effects was evaluated using repeated measures analysis of variance (ANOVA) as described below.

Release of Sfrp4 in vitro *and* in vivo

Serum was obtained from normal (no ischemic injury), control (ischemic injury followed by application of PBS or empty polyhedra particles), and Sfrp4-treated rats 1 or 10 weeks after control or Sfrp4 application (for fibrin glue samples there was a 2 week lag between ischemic injury and treatment). About 100 μL of rat serum was immobilized on the bottom of the ELISA plate, followed by detection with anti-Sfrp4 antibody (R&D System AF1827) and anti IgG-HRP (2nd antibody; R&D System, HAF019). The release of Sfrp4 from S-PH was estimated by fixing 5×10^3 or 5×10^4 cubes of S-PH to the bottom of 24-well plate wells followed by the determination of Sfrp4 concentration by ELISA after culture of 1.5×10^5 primary rat cardiomyocytes in the precoated wells with 500 μL of Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum.

Quantitative reverse transcriptase polymerase chain reaction

Total RNA from the LV myocardial tissue blocks was extracted with the RNeasy mini kit (QIAGEN). Quantitative reverse transcriptase (qRT)-PCR was performed with an ABI PRISM 7000 (Life Technologies) using SYBR Premix EX Taq™ (RR041A; Takara) in accordance with the manufacturer's instructions. Primers used are listed in Supplemental Table S2 (available online at www.liebertonline.com/ten). Expression measurements were normalized with respect to glyceraldehyde 3-phosphate dehydrogenase expression.

Immunohistological staining

Myocardial tissues were stained with a number of primary antibodies (Supplemental Table S2). Antibodies were observedwith the ABC detection kit (Vector Laboratories).

Cell proliferation was measured by *in vivo* BrdU incorporation. Ten mg of BrdU (Sigma) was administered to rats via intraperitoneal injection 3 days after LAD ligation and heart sections were prepared the next day (24 h labeling). BrdU incorporation was detected with the BrdU *In situ* Detection kit (BD Pharmingen; #550803). The number of BrdU(+) cells was determined in three randomly chosen areas (300×400 μm) lying in the ischemic border areas of three PBS- or Sfrp4-treated rats (total 9 areas).

The vascular density in infarcted border areas 3 days after LAD ligation was evaluated by scoring the number of von Willebrand Factor (vWF)-expressing vessel-like structures in nine randomly selected areas (300×400 μm) from three PBS- or Sfrp4-treated rats (three areas/rat). Frozen sections were stained with anti-human vWF antibody (SIGMA: A-254, 1:100 dilution) followed by detection with donkey anti-rabbit IgG peroxidase-linked-specific F(ab')2 fragment (GE Healthcare:NA9340, 1:100 dilution). The peroxidase substrate kit DAB (VECTOR:SK-4100) was used for the detection of vWF. Nuclei were stained with Mayer's hemalum solution (E.MERCK:1.09249.0500) for microscopic observation.

Measurement of left ventricle wall thickness and percent fibrosis

Horizontally sectioned rat heart slices were stained by Masson's Trichrome. The wall thickness of sectioned hearts in ischemic area was measured in a blind fashion at 4 different points in a total of 10 rats (4 points/rat) for each treatment group. The percentage of fibrous area in ischemic regions was determined by calculating the area of positive Masson's Trichrome staining region (blue) as a proportion of the total ischemic area utilizing an Adobe Photoshop (www.adobe.com) two-value recognition function. The proportion of fibrous area was measured in four rats for each treatment group.

Statistical analysis

All statistical analyses were performed using the indicated functions and packages in the R software environment (www.r-project.org).

EF data

All EF data were normalized by preinjury values (i.e., expressed as a fraction of the preinjury EF for that rat) to minimize noise caused by differences between individual rats. Postinjury EFs and standard errors are shown in Supplemental Fig. S1 (available online at www.liebertonline.com/ten). Individually normalized EF data were then analyzed using repeated measures ANOVA as implemented in the "ANOVA" function of the "car" R package. We used the "ANOVA" function to test for the presence of treatment and treatment–time interaction effects. The "ANOVA" function performs both type II repeated measures multivariate ANOVA and univariate type II repeated-measures ANOVA with corrections for departures from sphericity. Since the inference of *F*-values from multivariate ANOVA is nontrivial, the "ANOVA" function reports 4 different *p*-value estimates based on different means of estimating the degrees of freedom within the data set. Hence, the "ANOVA" function reported 4 multivariate ANOVA *p*-value estimates (Pillai, Wilks, Hotelling-Lawley and Roy for treatment and treatment-time interaction) and 4 univariate *p*-value estimates (raw treatment and treatment-time interaction as well as two different corrections) (Greenhouse-Geisser and Huynh-Feldt) for departures from sphericity. In the cases where more than two treatment groups were present in the data set, we used models containing specific subsets (depending on the purpose of the experiment) of the data to determine significant differences between treatments. Although this could lead to type I errors, the *p*-values were sufficiently low to withstand Bonferroni corrections for multiple testing. All *p*-values are reported in Supplemental Table S3 (available online at www.liebertonline.com/ten).

We also performed one-tailed *t*-tests at all time points between specific treatment groups to indicate the times at which specific effects occur (Supplemental Fig. S2, available online at www.liebertonline.com/ten).

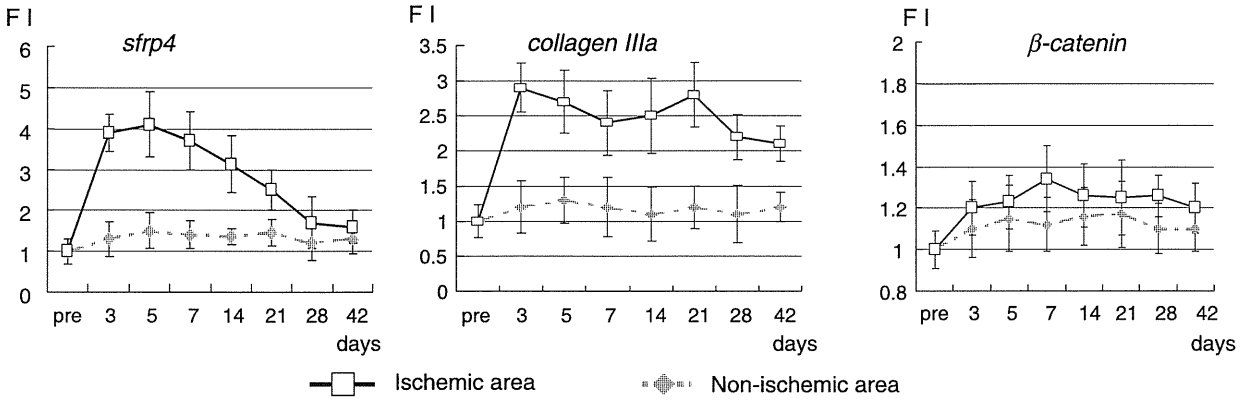


FIG. 1. Gene expression profile of ischemic-injury-related molecules. Heart tissues from nonischemic and border (ischemic/nonischemic) areas were prepared at, before (pre), or 3, 5, 7, 14, 21, 28, and 42 days after LAD ligation. Gene expression of *Sfrp4*, *collagen type IIIa*, or *β-catenin* was determined by qRT-PCR. The points and error bars show mean and standard deviations, respectively, of four rat heart samples obtained at the designated times. FI stands for fold induction of qRT-PCR values at designated times compared with that at pre. Sfrp4, secreted frizzled related protein 4; LAD, lateral anterior descendant; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction. Color images available online at www.liebertonline.com/ten.

Fibrosis and heart wall thickness

Wall thickness and fibrosis percentage mean values for individual treatment groups were analyzed by a one-way multivariate ANOVA with treatment groups specified as either control (PBS or empty polyhedra delivered as described) or Sfrp4 treated (soluble, or in polyhedra). The parameters of the negative linear relationship between fibrosis percentage and wall thickness were estimated using the R "lm" function.

In vivo *Sfrp4 release*

Due to the fragmented nature of the Sfrp4 release data, we performed a number of different univariate ANOVA analyses on specific subsets of the data to ask the pertinent questions (see Supplemental Fig. S3 for details, available online at www.liebertonline.com/ten). Although this could lead to the appearance of type I errors, the *p*-values obtained were sufficiently small to pass Bonferroni corrections.

Gene expression, cell proliferation, and vascular densities

These data sets were tested using paired *t*-tests as each one contained only one reasonable control versus experimental comparison.

Result

Sfrp4 transcription is upregulated in ischemic heart models

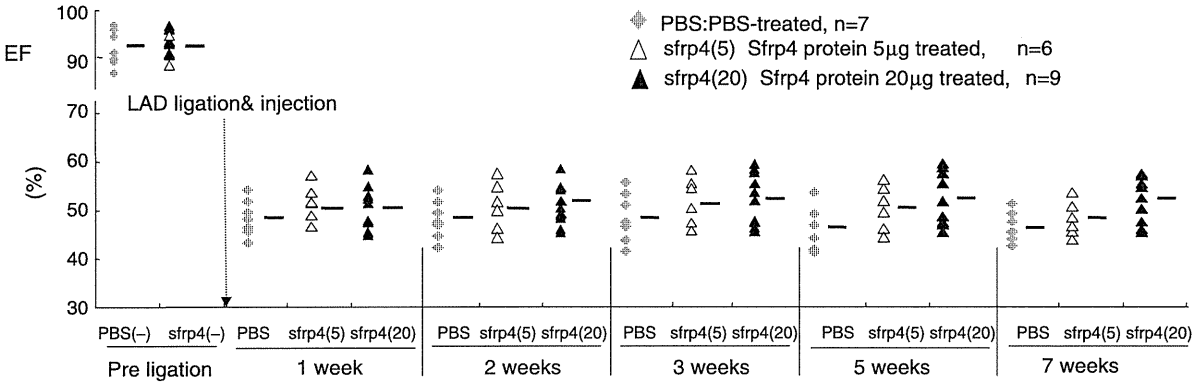
We first examined the expression patterns of rat *Sfrp4*, *β-catenin*, and *collagen type IIIa* in the ischemic border region of LAD-ligated rat hearts using qRT-PCR to confirm expression of these genes during wound healing (Fig. 1). As shown in Figure 1, the levels of *Sfrp4* transcripts were upregulated in rat ischemic heart, whereas expression of *β-catenin* was not markedly upregulated. The level of *collagen IIIa* transcripts remained elevated throughout the 6 weeks examined, suggesting an active tissue remodeling process that takes more than a month after heart ischemic injury.

IM administration of Sfrp4 recombinant protein improved cardiac function of ischemic heart

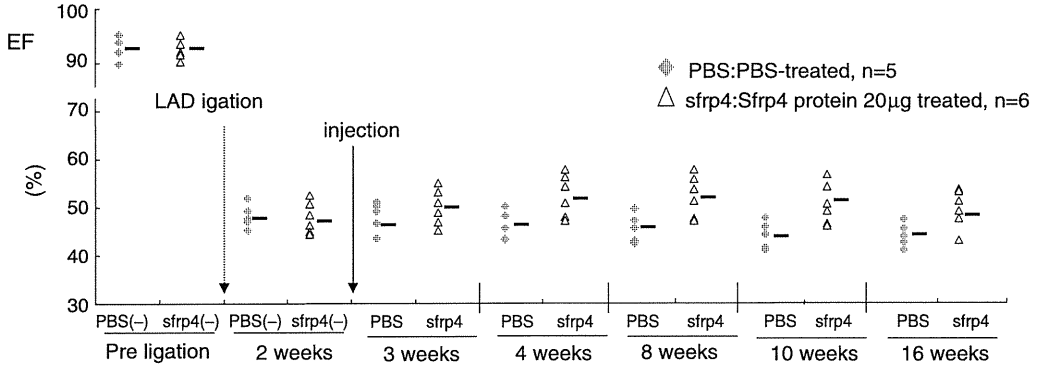
Sfrp4 recombinant protein was administered to the intracardiac musculature just after ischemic injury to evaluate the efficacy of administration of the agent in the acute phase. Further deterioration of cardiac function as determined by EF, FS (functional shortening), or fractional area change in the LAD ligation model (Supplemental Table S4, available online at www.liebertonline.com/ten) was blocked as a re-

FIG. 2. Sfrp4 recombinant protein exerts a cardio-protective effect. Cardiac function of PBS- or Sfrp4-treated rats as determined by EF before and after ischemic or recanalization injuries. EFs were monitored by echocardiography at the indicated time points and calculated as described in the Materials and Methods section. Other cardiac parameters, including fractional shortening (FS) and functional area change, are shown in Supplemental Table S4. (**A**) IM injection of Sfrp4 in an acute ischemic model. Ischemic hearts were generated by LAD ligation, and 100 μL of PBS ($n=7$), 5 μg Sfrp4 protein ($n=6$), or 20 μg Sfrp4 protein ($n=9$) was injected to the ischemic border zones soon after LAD ligation. (**B**) IM injection of Sfrp4 in a subacute ischemic model. About 100 μL of PBS ($n=5$) or 20 μg Sfrp4 protein ($n=6$) was administered intramuscularly 2 weeks after LAD ligation. (**C, D**) IM injection of Sfrp4 just after (**C**) or before (**D**) recanalization injury. About 100 μL PBS or 20 μg Sfrp4 protein was administered intramuscularly after or before a transient 1 h LAD ligation. All treatments except 5 μg Sfrp4 (**A**) show either significant ($p<0.05$) treatment or time-treatment interaction effects as judged by repeated measures ANOVA, and the effect of 20 μg Sfrp4 is different to that of 5 μg ($p=2.38E\text{-}4$). Summary measurements are shown in Supplemental Fig. S1 and *p*-values are reported in Supplemental Table S3. PBS, phosphate-buffered saline; EF, ejection fraction; IM, intramuscular; ANOVA, analysis of variance. Color images available online at www.liebertonline.com/ten.

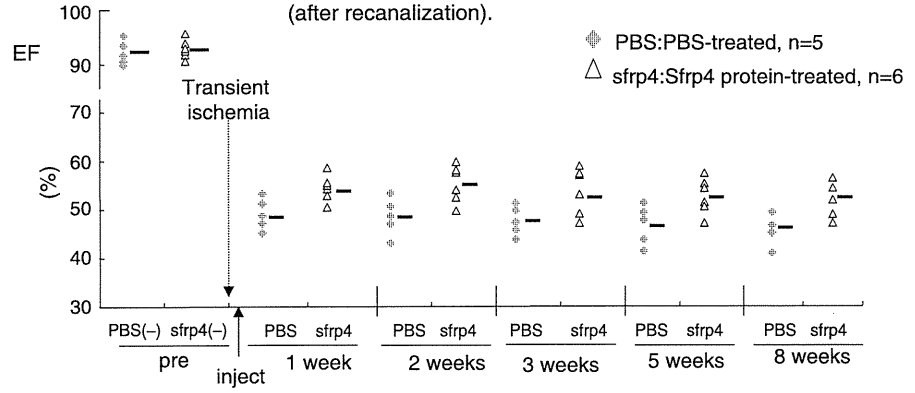
A Intra-muscular injection of Sfrp4 in an acute ischemic model.



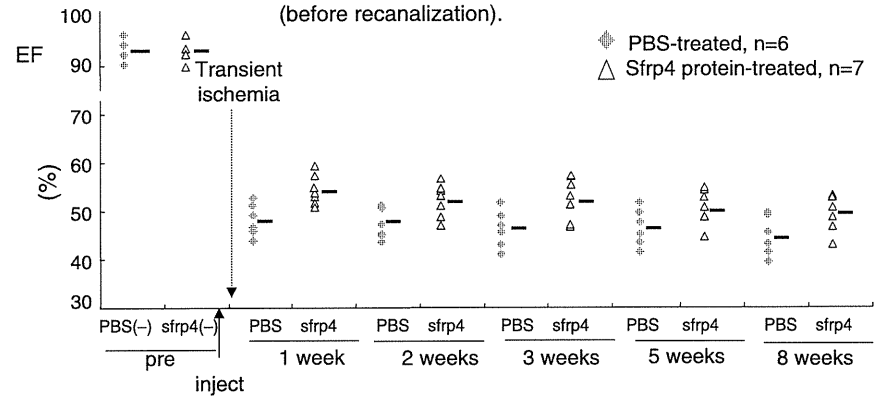
B Intra-muscle injection of sfrp4 in a sub-acute ischemic model



C Intra-musclular injection of Sfrp4 in a reperfusion injury model (after recanalization).



D Intra-musclular injection of Sfrp4 in a reperfusion injury model (before recanalization).



sult of administrating Sfrp4 protein just after ischemic injury in a dose-dependent manner (Fig. 2A).

We then examined the efficacy of Sfrp4 administration during the subacute phase (2 weeks after LAD ligation). Interestingly, IM Sfrp4 protein injection resulted in a partial recovery of cardiac function during the 3–10 weeks examined (Fig. 2B), suggesting that the tissue remodeling process continues for at least several weeks after the ischemic injury. Third, the therapeutic efficacy of Sfrp4 protein administration for the treatment of transient LAD ischemic attack was evaluated using a rat LAD recanalization model. Similarly, IM administration of 20 μg Sfrp4 protein both before and after LAD ligation improved cardiac function after reperfusion injury (Fig. 2C, D).

Sfrp4 in a slow-releasing polyhedra form has a longer and superior cardio-protective effect

As demonstrated in Figure 1, scar formation and the tissue remodeling process takes more than a month. Hence, we reasoned that administration of Sfrp4 in a long-lasting, slow-releasing form might exert a stronger therapeutic effect on ischemic heart recovery than simple injection. To examine this, we used polyhedron cubes to facilitate a slow release of Sfrp4. (Fig. 3A).[23] Release of the cargo protein from polyhedra cubes occurs as a result of the decay of the polyhedra cubes by proteases secreted from adjacent cells. Although we have not determined the optimum therapeutic dose for S-PH for this application, 5×10^3 cubes of S-PH emit 1.6 ng of Sfrp4 protein when incubated in the presence of primary rat cardiomyocytes for 4 days (Fig. 3B) in 500 μL culture medium, and we estimated that 2.5×10^6 polyhedra contain the equivalent of 20 μg of Sfrp4 protein (methods). We found that 2.5×10^6 cubes of S-PH block the deterioration of cardiac function when administered intramuscularly at the onset of ischemic injury, but we failed to detect any effect at a dose of 2.5×10^5 cubes (data not shown). Indeed, statistical analysis indicates that 20 μg of soluble Sfrp4 shows the strongest effect at around 8 weeks, but the beneficial effect drops rapidly after this time point (Fig. 3C; Supplemental Figs. S1 and S2). On the other hand, administration of 2.5×10^6 cubes of S-PH provided a longer therapeutic effect than that of 20 μg of Sfrp4 recombinant protein especially at 8–20 weeks after LAD ligation, although its effect also appears to drop after 8 weeks (Fig. 3C; Supplemental Figs. S1 and S2).

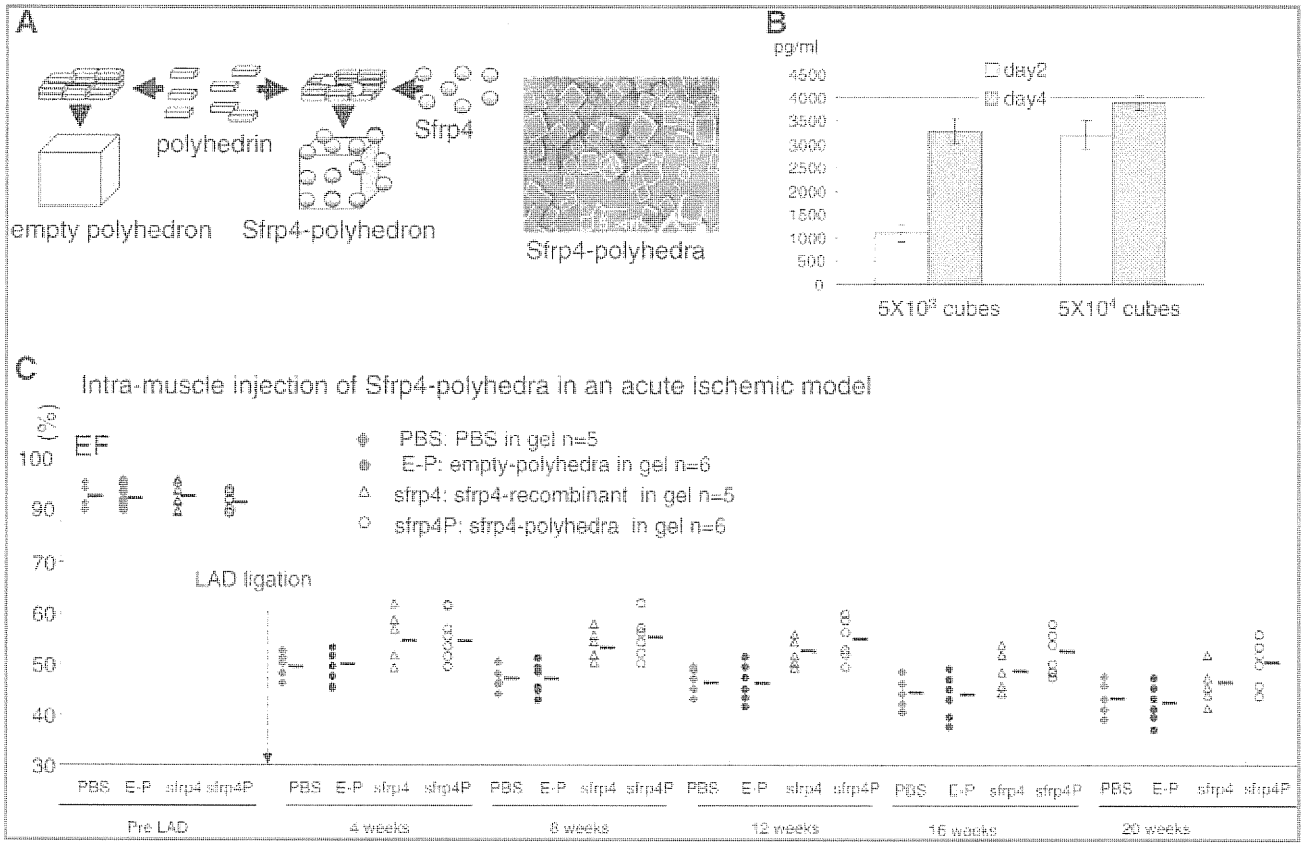


FIG. 3. Administration of Sfrp4 in a slow-releasing cube polyhedra form demonstrated long-term therapeutic effects. (**A**) Schema for generation of empty polyhedra and Sfrp4 immobilized in polyhedra (S-PH) and scanning electron microscopic image of S-PH. (**B**) Concentration of Sfrp4 in the culture medium after culture of primary rat cardiomyocytes in the presence of S-PH ($n=3$) for 2 or 4 days as determined by ELISA. Mean values and standard deviations are indicated. (**C**) Twenty microliter PBS ($n=5$), 2.5×10^6 cubes of empty polyhedra ($n=6$), 20 μg Sfrp4 protein ($n=5$), or 2.5×10^6 cubes of S-PH ($n=6$) were administered just after LAD ligation. EFs of indicated groups are shown. The full data set contains significant treatment effects as determined by repeated measures ANOVA ($p=1.1e\text{-}4$). Significant ($p=0.016$) time–treatment interaction effects were also observed between polyhedra bound and soluble Sfrp4. Complete set of p-values are shown in Supplemental Table S3. S-PH, Sfrp4 containing polyhedra. Color images available online at www.liebertonline.com/ten.

ELISA measurements of serum Sfrp4 concentrations up to 10 weeks after Sfrp4 administration indicated small increases in Sfrp4 levels that appear to be approximately correlated with the efficacy of the treatment methods (Supplemental Fig. S3).

S-PH can be applied in combination with a biodegradable vehicle

The size of the rat heart makes it feasible to administer therapeutic agents by IM injection; however, the large size of the human heart may make it difficult to evenly distribute Sfrp4 protein across the ischemic region by simple injection. Collagen sheets have previously been used to facilitate wound healing after serious burn and other types of skin injury.[24] Collagen sheets show low immunoreactivity and are degraded in tissues after a period of months. Since polyhedra are resistant to dessication they can easily be immobilized onto the surface of collagen sheets, and this might provide a means to attain a uniformly localized and enduring drug application to a large injured area. S-PH immobilized on collagen type I sheets were applied to ischemic areas of the heart (Fig. 4A, B) just after LAD ligation. This intervention provided a similar level of protection against heart dysfunction to that seen by straight injection of polyhedra (Fig. 4C). Histological examination showed neither collagen type I sheet nor polyhedra cubes in heart sections 10 weeks after LAD ligation (data not shown), suggesting that both the collagen sheet and polyhedra were absorbed in the peri-cardiac cavity.

Alternatively, fibrin glue might provide a useful means of applying polyhedra to larger ischemic areas. Fibrin glue is presently used in a number of surgical applications and as such has been validated for clinical use.[25] Hence, we also tried plastering S-PH in fibrin glue to ischemic hearts 2 weeks after LAD ligation, as a model for the treatment of patients in the subacute phase who have not received treatment at the onset of ischemic attack. S-PH in fibrin glue (Boveal) were plastered precisely to the ischemic heart area following a reopening of the chest cavity 2 weeks after LAD

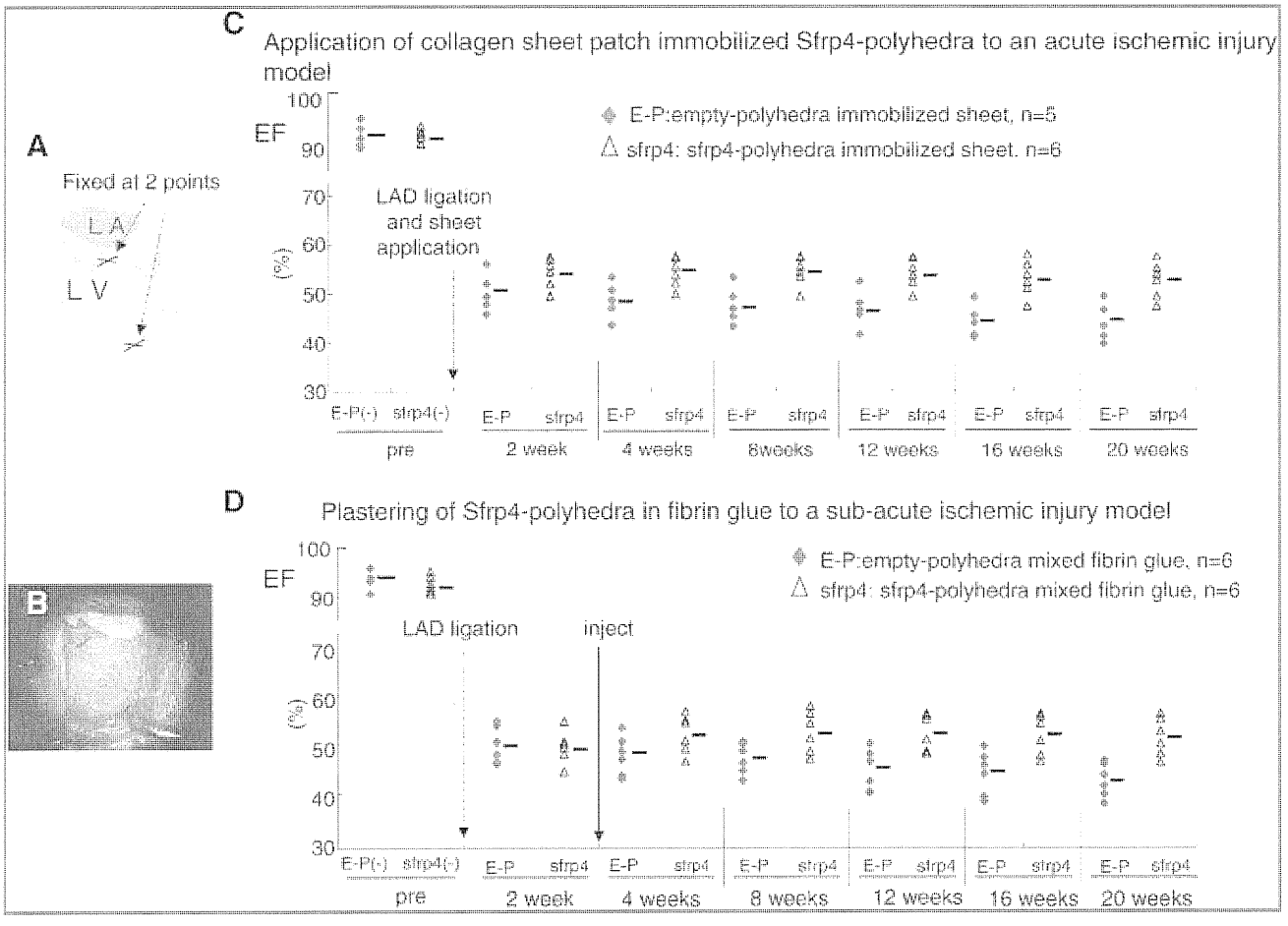


FIG. 4. Sfrp4 polyhedra applied with a range of vehicles ameliorated cardiac dysfunction caused by ischemic injury. (**A**) Schema for application of collagen sheet to ischemic left ventricle after LAD ligation. (**B**) Application of collagen sheet during operation. (**C**) About 5.0×10^6 cubes of empty polyhedra ($n=5$) or S-PH ($n=6$) were immobilized on collagen type I sheets by air-drying and applied to the ischemic region of rat hearts just after LAD ligation. (**D**) About 5.0×10^6 cubes of empty polyhedra ($n=6$) or S-PHs ($n=6$) were mixed with 150 μL of fibrin glue and plastered to ischemic regions of rat hearts 2 weeks after LAD ligation by reopening of the chest. EFs of respective group at designated time points are shown in the graphs. The use of both collagen sheet ($p=2.96\text{e-}3$) and fibrin glue ($p=3.5\text{e-}3$) provided statistically significant effects as judged by repeated measures ANOVA.