Generation of disease-specific induced pluripotent stem cells from patients with rheumatoid arthritis and osteoarthritis

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Abstract

Introduction: Since the concept of reprogramming mature somatic cells to generate induced pluripotent stem cells (iPSCs) was demonstrated in 2006, iPSCs have become a potential substitute for embryonic stem cells (ESCs) given their pluripotency and "stemness" characteristics, which resemble those of ESCs. We investigated to reprogram fibroblast-like synoviocytes (FLSs) from patients with rheumatoid arthritis (RA) and osteoarthritis (OA) to generate iPSCs using a 4-in-1 lentiviral vector system.

Methods: A 4-in-1 lentiviral vector containing Oct4, Sox2, Klf4, and c-Myc was transduced into RA and OA FLSs isolated from the synovia of two RA patients and two OA patients. Immunohistochemical staining and real-time PCR studies were performed to demonstrate the pluripotency of iPSCs. Chromosomal abnormalities were determined based on the karyotype. SCID-beige mice were injected with iPSCs and sacrificed to test for teratoma formation.

Results: After 14 days of transduction using the 4-in-1 lentiviral vector, RA FLSs and OA FLSs were transformed into spherical shapes that resembled embryonic stem cell colonies. Colonies were picked and cultivated on matrigel plates to produce iPSC lines. Real-time PCR of RA and OA iPSCs detected positive markers of pluripotency. Immunohistochemical staining tests with Nanog, Oct4, Sox2, Tra-1-80, Tra-1-60, and SSEA-4 were also positive. Teratomas that comprised three compartments of ectoderm, mesoderm, and endoderm were formed at the injection sites of iPSCs. Established iPSCs were shown to be compatible by karyotyping. Finally, we confirmed that the patient-derived iPSCs were able to differentiate into osteoblast, which was shown by an osteoimage mineralization assay.

Conclusion: FLSs derived from RA and OA could be cell resources for iPSC reprogramming. Disease- and patient-specific iPSCs have the potential to be applied in clinical settings as source materials for molecular diagnosis and regenerative therapy.

Introduction

The concept of reprogramming mature somatic cells to generate induced pluripotent stem cells (iPSCs) was demonstrated by Takahashi and Yamanaka in 2006 [1]. Four factors, namely Oct4, Klf4, Sox2, and c-Myc, were transduced into somatic cells to reprogram and generate iPSCs.

Subsequently, iPSCs have become a potential substitute for embryonic stem cells (ESCs) given their pluripotency and stemness characteristics, which resemble those of ESCs [2,3]. iPSCs may have important potential clinical applications as drug screening platforms, in pathophysiological studies in dishes, and as candidate cell sources for regenerative medicine [4-7].

The iPSCs used in pathophysiological studies in dishes were generated from the primary cells that originated from patients with neurological, hematological, metabolic, cardiovascular, primary immunodeficiency diseases, and so on [5,8-10]. These pioneering studies have detected many novel pathophysiological mechanisms, which were impossible to study previously because of the inaccessibility of...
disease tissues and cells. Patient-specific iPSCs are particularly useful for studying diseases with complex mechanisms, which are affected by several factors that range from the genetic background to environmental modifications.

Rheumatoid arthritis (RA) may be a promising target disease for iPSC applications because of its complex pathophysiology. The iPSCs from RA patients could be extended to a regenerative approach via their differentiation into mature chondrocytes and osteocytes, which synthesize cartilage and bone. We therefore selected fibroblast-like synoviocytes (FLSs) from RA and osteoarthritis (OA) for reprogramming using a four-in-one lentiviral vector, which contained four factors: Oct4, Klf4, Sox2, and c-Myc. RA FLSs, which are regarded as major pathophysiological players in RA, are thought to be a good candidate for reprogramming to simulate the disease RA in dishes [11-13].

In this study, we successfully reprogrammed RA FLSs and OA FLSs to generate disease-specific iPSCs. Their pluripotency was demonstrated by immunohistochemical staining and teratoma formation in vivo. To our knowledge, this is the first attempt to induce stem cells from RA FLSs.

Methods

Patient recruitment and synovia preparation

Four patients were used for synovia preparation. RA patients (n = 2) who satisfied the 1987 revised criteria of the American College of Rheumatology (formerly the American Rheumatism Association) [14] and OA patients (n = 2) were recruited from the outpatient clinic at the Department of Rheumatology, Seoul St. Mary's Hospital, Seoul, South Korea. These patients had received arthroscopic synovectomy or total knee replacement surgery. Synovia samples were obtained during the operations. Eligibility for OA inclusion required that individuals had primary knee OA, which was diagnosed according to American College of Rheumatology criteria. The experimental protocol was approved by the Catholic University of Korea Human Research Ethics Committee.

Isolation and maintenance of RA and OA fibroblast-like synoviocytes

RA and OA synovia were stored in the sample bank of the Rheumatism Research Center, Seoul, South Korea. These samples were classified only by disease and stored in a blind manner. The institutional review board permitted that a patient consent form was not necessary because samples were anonymous and did not contain patient information. Tissues were homogenized and resuspended in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco by Invitrogen, Carlsbad, CA, USA) containing 0.01% collagenase and were incubated for 4 hours at 37°C with vigorous shaking. Cells were washed and resuspended in DMEM supplemented with 20% fetal bovine serum (FBS) (Gibco by Invitrogen), and 1% penicillin/streptomycin solution (Gibco by Invitrogen). Cells were cultured until the adherent fibroblast cells achieved confluence.

Lentivirus production and transduction

293FT cells (Invitrogen, Carlsbad, CA, USA) were plated at 80% confluence in 100-mm dishes and transfected with 12 μg of four-in-one reprogramming plasmid (Oct4, Sox2, Klf4, and c-Myc), 9 μg packaging pPAX2 plasmids, and 3 μg pMD2G plasmids using Lipofectamine 2000 (Invitrogen). After about 48 to 72 hours of culture, the virus was harvested and mixed with Lenti-X Concentrator (Clontech Laboratories, Mountain View, CA, USA). After overnight incubation at 4°C, viruses were precipitated at 1,500 × g and resuspended in phosphate-buffered saline. For virus infection, RA or OA FLSs were seeded onto six-well plates. The lentivirus was applied with culture medium for overnight infection. The iPSC colonies were picked after 18 to 20 days of reprogramming.

Cell culture and maintenance of patient-specific iPSCs

RA or OA FLSs were maintained in DMEM containing 20% FBS at 37°C, with 95% air and 5% CO2 in a humidified incubator. All of the cells used for reprogramming were at passage 8. Patient-specific iPSCs were maintained in Matrigel-coated tissue culture dishes (BD Biosciences, San Jose, CA, USA) with E8 human ESC medium.

Quantitative real-time polymerase chain reaction

Total RNA was isolated using an RNasy Plus Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcriptase polymerase chain reaction was performed using an iScript™ cDNA Synthesis Kit (BIORAD, Marnes-La-Coquette, France). Gene expression was quantified by SYBR Green real-time polymerase chain reaction using an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The relative mRNA levels were normalized to the values of GAPDH mRNA for each reaction. The primer sequences are described in Additional file 1.

Immunostaining

The iPSC clones were fixed with 4% paraformaldehyde and immunostaining was performed using the following primary antibodies: SSEA-4, Tra-1-60 and Tra-1-80 (Millipore, Billerica, MA, USA), Oct3/4 and Nanog (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Sox2 (BioLegend, San Diego, CA, USA). Samples were incubated with Alexa Fluor 594-conjugates or 488-conjugated secondary antibody (Invitrogen) and detected by indirect immunofluorescence microscopy.
Teratoma formation
Teratoma formation was performed and analyzed with the approval of the Institutional Animal Care and Use Committee of Applied StemCell (protocol number APP-12-001-Y2; Sunnyvale, CA, USA). Briefly, undifferentiated iPSCs (1 × 10^6) were suspended in 10 μl Matrigel (BD Biosciences) and delivered using a 28.5 gauge syringe into the subrenal capsule of 8-week-old SCID-beige mice. Eight weeks after cell delivery, the tumors were explanted and subjected to hematoxylin and eosin staining.

Osteogenic differentiation
For osteogenic differentiation, we cultured iPSCs in osteogenic differentiation medium (ODM) as described by Kao and colleagues [15]. ODM is DMEM supplemented with 15% FBS, 50 μg/ml ascorbate-2-phosphate, 10 nmol/l dexamethasone, and 10 mmol/l β-glycerophosphate. At day 7 after osteogenic induction, in vitro mineralization of cells was assessed using the OsteoImage Mineralization Assay kit (Lonza, Basel, Switzerland) according to the manufacturer’s manual. Fluorescent signals from the hydroxyapatite portion were detected by fluorescence microscope (Axio observer 2.1; ZEISS, Thornwood, NY 10594, USA).

Karyotyping
We added 30 μl Chromosome Resolution Additive (Genial Genetic Solutions Ltd, Runcorn, UK) to each six-well plate. After 1 hour of incubation, colcemid® was treated for 30 minutes. Cells were harvested using trypsin and treated by prewarmed hypotonic solution (KCl). Fixation was then performed with 1:3 acetic acid:methanol solution and the slide was prepared for chromosome analysis. The chromosome analysis was performed using a trypsin-Giemsa banding technique. At least 20 metaphases were analyzed.
Figure 2 (See legend on next page.)
Ethics statement
This study protocol was approved by the institutional review board of The Catholic University of Korea (KC12T1SI0861).

Results
Isolation of RA and OA fibroblast-like synoviocytes and successful generation of disease-specific iPSCs
We cultured FLSs from patients with RA ($n=2$) and with OA ($n=2$) (Figure 1A). The four-in-one vector was transduced into RA FLSs and OA FLSs, and they were serially observed. Colonies were formed and picked manually after 18 to 20 days. They were cultured through five passages (Figure 1B and 1C).

Positive expression of pluripotency markers by RA and OA iPSCs
RA and OA iPSCs were immunostained against Nanog, Oct4, Sox2, Tra-1-80, Tra-1-60, and SSEA-4. These factors all stained positively in six iPSC clones that originated from two RA patients and two OA patients (Figure 2B, 2C and Additional file 2). The pluripotency mRNA markers for Nanog, Oct4, Sox2, Klf4, and Rex also increased, according to the real-time reverse transcriptase polymerase chain reaction analysis (Figure 2A). Interestingly, the level of Klf4 was elevated before reprogramming in RA FLSs and OA FLSs. Other factors began to increase after reprogramming and they peaked at higher levels than those found in H7 cells.

Successful teratoma formation and normal karyotyping
The RA iPSC karyotype had a normal chromosomal pattern of 44 + XX (Figure 3A). RA iPSCs were injected into the kidney capsule and testis of SCID mice. At 12 weeks, teratomas formed successfully and they comprised three compartments: ectoderm, mesoderm, and endoderm (Figure 3B). Each compartment contained glandular tissue, connective tissue, blood vessels, adipose tissue, and skin-like structures.

Osteogenic differentiation of RA and OA iPSCs
To confirm the osteogenic capability of patient-derived iPSCs, we tried to differentiate RA and OA patient-specific iPSCs to osteocytes. RA iPSCs and OA iPSCs were incubated in ODM to induce osteogenic differentiation (Figure 4). After 7 days of osteogenic induction, in vitro mineralization of cells was assessed by the OsteoImage Mineralization Assay Kit (Lonz). Fluorescent signals for mineralization were detected in RA iPSCs and OA iPSCs incubated with ODM, which means osteogenic cells were successfully differentiated from these iPSCs. This result shows that patient-derived iPSCs have a potential for bone formation.

Discussion
Insights into the pathophysiology of RA have been derived largely from animal models such as collagen-induced arthritis, collagen antibody-induced arthritis, IL-1RaKO mice, and KBX/N serum transfer mice. However, the existing arthritic mouse model is not fully representative of the actual pathophysiology in patients with RA [16,17]. Indeed, it has been frequently suggested that insights from mouse models have rarely translated successfully to the clinic [18]. The production of simulation platforms and better access to genetically predisposed human cells may allow scientists to study the pathophysiology of diseases with greater ease. Previously, it was impossible to perform in vitro mechanistic studies and to produce in vivo transplant systems. The biological status obtained in simulation platforms using patient-derived iPSCs is now considered more representative of a disease than other systems. We thus generated human iPSCs from patients with RA ($n=2$) and with OA ($n=2$). Moreover, the osteogenic potential of RA iPSCs and OA iPSCs was shown by producing hydroxyapatite after stimulation with osteogenic differentiation. These results mean that patient-derived iPSCs have a potential of differentiation into tissues like other stem cells. We are going to investigate that these RA iPSCs and OA iPSCs could be suitable candidate materials for disease modeling and drug screening platforms.

The generation of disease-specific iPSCs has been attempted for many diseases [5,19-23]. The initial target diseases of iPSC generation were genetic deficiency diseases such as congenital anomalies or familial inherited diseases. Recently, iPSC studies have been extended to chronic diseases where the genetic etiology plays a less significant role than was the case in diseases studied
previously. These diseases have mixed pathophysiological mechanisms with genetic background and environmental effects. The progression of RA is explained by a combination of susceptible genetic background and environmental exposure to *Porphyromonas gingivalis*, smoking, and other factors [24,25]. We thus consider that disease modeling may be applicable to RA after the generation of disease-specific iPSCs. Various environmental factors could be used to challenge an RA-simulated platform using iPSCs in a more sophisticated and individual-specific manner.

RA is characterized by its autoimmune mechanism, chronic inflammation, cartilage erosion, and bone destruction. In the final stage, the joints become dysfunctional. The destruction of cartilage and bone are the main causes of dysfunctionality and many scientists have tried to restore the defective areas. However, this approach has met with little success because of the cell sources used and immune rejection. Patient-specific iPSCs are regarded as good candidates for regeneration therapy because they may escape immunological rejection and exhibit pluripotency to differentiate into target cells such as cartilage and bone [13,26]. However, several issues need to be overcome before *in vivo* applications. Every material that is applied to humans needs to satisfy the rules of good clinical practice. In particular, good clinical practice guidelines prohibit the use of animal-derived products and oncogenic material, which is why the clinical application of iPSCs will...
require more time. Intensive efforts are ongoing to develop iPSCs using FBS-free media, feeder-free cultivation, and integration-free reprogramming, and numerous modifications are required to the protocol used for differentiation from iPSCs [27].

Two of the Yamanaka factors, c-myc and klf4, are regarded as oncogenes. Several modifications have been attempted to minimize the expression of these genes such as changing c-myc to n-myc and other combinations of factors. Interestingly, some somatic cells already expressed Klf4 and c-Myc, and therefore only required Sox2 and Oct4 for reprogramming [28]. In our experiments, RA and OA FLSs expressed Klf4 at a level that was suitable for establishing H7 stem cells. We thus expect that RA FLSs could be reprogrammed into iPSCs using minimized conditions, which would facilitate the minimal usage of oncogenes such as c-myc and klf4.

Occasionally, disease-specific iPSCs exhibit altered biological functions compared with ESCs, such as delayed cell proliferation and poor differentiation quality [2,3,29]. These phenomena were attributed to genuine problems with the primary cells that were reprogrammed. The disease state and drugs may thus affect the condition of primary cells. Our iPSCs had the full characteristics of stem cells according to immunostaining, karyotyping, and teratoma assays, but they exhibited slight alterations in their biological behavior. As various cytotoxic drugs such as methotrexate and other immunosuppressants are used in RA treatment, there is a significant possibility of prolonged exposure to cytotoxic drugs by RA FLSs, which could affect the reprogramming process. Further studies are needed to clarify the relationship between the quality of iPSCs and the primary somatic cells subjected to reprogramming.

Conclusions

FLSs derived from RA and OA could be cell resources for iPSC reprogramming. Disease-specific and patient-specific iPSCs have the potential to be applied in clinical settings as source materials for molecular diagnosis and regenerative therapy. A molecular diagnostic approach, by disease in a dish using patient-specific iPSCs, may reveal complicated and individualized pathophysiology of RA. Regenerated tissue from patients’ own iPSCs will be a good material for tissue repair and structural restoration.

Additional files

Additional file 1: Is a table presenting the primer sequences used in the polymerase chain reaction.
Additional file 2: Is a figure showing positive expression of pluripotency markers on OA and RA iPSCs. Other clones were generated during the reprogramming process. Immunofluorescence staining against Nanog, Oct4, Sox2, Tra-1-80, Tra-1-60, and SSEA-4 in RA and OA iPSCs. RA and OA iPSCs expressed high level of these markers.

Abbreviations

DMEM: Dulbecco’s modified Eagle’s medium; ESC: embryonic stem cell; FBS: fetal bovine serum; FLS: fibroblast-like synoviocyte; iPSC: induced pluripotent stem cell; OA: osteoarthritis; ODM: osteogenic differentiation medium; RA: rheumatoid arthritis; SCID: severe combined immune deficiency.

Competing interest

The authors declare that they have no competing interests.

Authors’ contributions

JL, YK, HY and JHJ carried out the molecular studies, cultured cells and drafted the manuscript. HJ carried out the immunohistochemical staining. JK and YAR performed the teratoma assay. MK and YGK participated in the design of the study. JHJ and SD conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

Authors’ information

JHJ is a visiting scholar at Stanford University and has participated in the project of reprogramming somatic cells to iPSCs. JHJ worked with JL and SD at the Stem Cell Institute of Stanford University. JHJ extended iPSC techniques to the rheumatologic field. Application of reprogramming techniques on RA FLSs to iPSCs was performed at The Catholic University of Korea. Karyotyping and teratoma formation were followed by the colleagues of Seoul St. Mary’s Hospital.

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1. Sakaguchi Y, Yamana K: Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006, 126:633–676.

2. Puri MC, Nagy A: Concise review: Embryonic stem cells versus induced pluripotent stem cells: the game is on. Stem Cells 2012, 30:10–14.

3. Ilic I, Izsinska Belmonte JC: Concise review: Induced pluripotent stem cells versus embryonic stem cells: close enough or yet too far apart? Stem Cells 2012, 30:333–41.

4. Tiscornia G, Vivas EL, Izpisua Belmonte JC: Diseases in a dish: modeling human genetic disorders using induced pluripotent cells. Nat Med 2011, 17:1570–1576.

5. Grizovic M, Javahanir A, Strulovic B, Daley GQ: Induced pluripotent stem cells – opportunities for disease modelling and drug discovery. Nat Rev Drug Discov 2011, 10:915–929.

6. Estes BT, Diekman BO, Gimble JM, Gulak F: Isolation of adipose-derived stem cells and their induction to a chondrogenic phenotype. Nat Protoc 2010, 5:1294–1311.

7. Choi KD, Vodyanik M, Slukvin II: Isolation of adipose-derived mesenchymal stem cells from human pluripotent stem cells. Nat Protoc 2011, 6:296–315.

8. Robinton DA, Daley GQ: The promise of induced pluripotent stem cells in research and therapy. Nature 2012, 481:295–305.

9. Oh Y, Wei H, Ma D, Liew R: Clinical applications of patient-specific induced pluripotent stem cells in cardiovascular medicine. Heart 2012, 98:443–449.

10. Onder TT, Daley GQ: New lessons learned from disease modelling with induced pluripotent stem cells. Curr Open Genet Dev 2012, 22:500–508.

11. Shirasawa S, Sekiya I, Sakaguchi Y, Yagishita K, Ichinose S, Muneta T: In vitro chordogenesis of human synovium-derived mesenchymal stem cells: optimal condition and comparison with bone marrow-derived cells. J Cell Biochem 2006, 97:84–97.

12. Sakaguchi Y, Sekiya I, Yagishita K, Muneta T: Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. Arthritis Rheum 2005, 52:2521–2529.

13. Kim MJ, Son MJ, Son MY, Seol B, Kim J, Park J, Kim JH, Kim YH, Park SA, Lee CH, Lee KS, Han YM, Chang JS, Cho YS: Generation of human induced pluripotent stem cells from osteoarthritis patient-derived synovial cells. Arthritis Rheum 2011, 63:3010–3021.

14. Annett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988, 31:315–324.

15. Kao CL, Tai LH, Chou SH, Chen YJ, Lee KH, Chou SJ, Chang YL, Cheng CM, Chen SJ, Ku HH, Li HF: Resveratrol promotes osteogenic differentiation and protects against dexamethasone damage in murine induced pluripotent stem cells. Stem Cells Dev 2010, 19:247–258.

16. Hegen M, Keith JC, Jr, Collins M, Nickerson-Nutter CL: Utility of animal models for identification of potential therapeutic agents for rheumatoid arthritis. Ann Rheum Dis 2008, 67:1505–1515.

17. Vietboom MP, Jonger M, Tak PP, Hart AB: Preclinical models of arthritic disease in non-human primates. Drug Discov Today 2007, 12:327–335.