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Human Sclera Maintains Common Characteristics with Cartilage throughout Evolution

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Abstract

Background: The sclera maintains and protects the eye ball, which receives visual inputs. Although the sclera does not contribute significantly to visual perception, scleral diseases such as refractory scleritis, scleral perforation and pathological myopia are considered incurable or difficult to cure. The aim of this study is to identify characteristics of the human sclera as one of the connective tissues derived from the neural crest and mesoderm.

Methodology/Principal Findings: We have demonstrated microarray data of cultured human infant scleral cells. Hierarchical clustering was performed to group scleral cells and other mesenchymal cells into subcategories. Hierarchical clustering analysis showed similarity between scleral cells and auricular cartilage-derived cells. Cultured micromasses of scleral cells exposed to TGF-βs and BMP2 produced an abundant matrix. The expression of cartilage-associated genes, such as Indian hedge hog, type X collagen, and MMP13, was up-regulated within 3 weeks in vitro. These results suggest that human 'sclera'-derived cells can be considered chondrocytes when cultured ex vivo.

Conclusions/Significance: Our present study shows a chondrogenic potential of human sclera. Interestingly, the sclera of certain vertebrates, such as birds and fish, is composed of hyaline cartilage. Although the human sclera is not a cartilaginous tissue, the human sclera maintains chondrogenic potential throughout evolution. In addition, our findings directly explain an enigma that the sclera and the joint cartilage are common targets of inflammatory cells in rheumatic arthritis. The present global gene expression database will contribute to the clarification of the pathogenesis of developmental diseases such as high myopia.

Citation: Seko Y, Azuma N, Takahashi Y, Makino H, Morito T, et al. (2008) Human Sclera Maintains Common Characteristics with Cartilage throughout Evolution. PLoS ONE 3(11): e3709. doi:10.1371/journal.pone.0003709

Editor: Che John Connon, University of Reading, United Kingdom

Received July 31, 2008; **Accepted** October 8, 2008; **Published** November 12, 2008

Funding: This study was supported by Research Fellowships of the Japan Society for the Promotion of Science (JSPS) for Young Scientists; a grant from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan and Health and Labor Sciences Research Grants; by a Research grant on Health Science Focusing on Drug Innovation from the Japan Health Science Foundation; by the Program for Promotion of Fundamental Studies in Health Science of the Pharmaceuticals and Medical Devices Agency; by a grant from the Terumo Life Science Foundation; by a Research Grant for Cardiovascular Disease from the Ministry of Health, Labor and Welfare; and by a Grant for Child Health and Development from the Ministry of Health, Labor and Welfare.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The eye receives information from the outside as the retinal image, converting it into electrical signals for the brain, leading to visual perception. The retinal image is stabilized by the balance of intraocular pressure and the curvatures of the scleral and corneal envelope. In order to keep this balance, the rigidity of the sclera and the cornea are essential, especially the sclera must be rigid enough for the eyeball to be rotated by powerful extraocular muscles adhering to the sclera. The sclera and the corneal stroma that are anatomically continuous have common characteristics such as mechanical rigidity, and share a common origin, i.e., the neural crest. However, the cornea and the sclera are different in transparency: the cornea is completely transparent to produce a sharp image on the retina; the sclera is opaque to avoid the internal light scattering affecting the retinal image. This corneal transparency has been attributed to significant changes in the structure, especially of collagen fibrils, in the latter stages of development [1]. Multipotent progenitor/precursor cells of corneal stroma are identified from the mouse eye [2]. On the other hand, existence of multipotent progenitor/precursor cells in the sclera remains unclarified. Although the sclera does not contribute significantly to visual perception, scleral diseases such as refractory scleritis, scleral perforation and pathological myopia are considered incurable or difficult to cure.

Microarray analysis of murine scleral development [3] and global sequencing analysis from the human scleral cDNA library [4] have been reported. To clarify pathogenesis of developmental diseases such as high myopia, a database of genes expressed in the sclera of younger donors is important. We here demonstrate with a global expression database of human infant sclera that the sclera derived from the neural crest evolutionarily retains characteristics of cartilage.

Results

Isolation and cell culture of human scleral cells

Scleral tissues were excised from surgical specimens collected during treatment for retinoblastoma. The scleral tissue was cut into smaller pieces and cultured in the growth medium. The scleral cells began growing out almost one week after the start of cultivation. Scleral cells exhibited a fibroblast-like spindle shape or polygonal shape in morphology when cultured in monolayer (Fig. 1A). The cells from PD 5 to PD 31 rapidly proliferated in culture, and propagated continuously (Fig. 1B). The cells stopped replicating and became broad and flat at PD 43 or 264 days, indicating that they had entered senescence. The morphological changes are PD-dependent.

Global outlook by hierarchical clustering and PCA

To clarify the specific gene expression profile of scleral cells, we compared the expression levels of 54,675 probes in the cultured scleral cells and other cultured cells (Table 1) using the Affymetrix GeneChip oligonucleotide arrays. We first performed hierarchical clustering and PCA on the expression pattern. PCA showed similarity between scleral cells and chondrocytes derived from elastic cartilage (Fig. 2A). Hierarchical clustering analysis based on all probes showed similarity between scleral cells and chondrocytes (Fig. 2B). This similarity led us to hypothesize that the scleral cells are chondrocytes when proliferated ex vivo, or have a chondrogenic potential. We then performed PCA from the expression data of cartilage-associated genes, including aggrecan, Sox9, and parathyroid hormone receptor (Table S1). These genes are categorized as "cartilage condensation" or "proteoglycan biosynthesis" according to Gene Ontology. PCA based on cartilage-associated genes demonstrated that scleral cells are grouped into the same category that includes chondrocytes, synovial cells, and synovial fluid-derived cells (Fig. 2C). The synovial cells and synovial fluid-derived cells used in this study have a strong chondrogenic potential [5–7]. Hierarchical clustering analysis based on the cartilage-associated genes also demonstrated that sclera, cartilage, synovium, and joint fluid are categorized into the same group (Fig. 2D, Fig. 2E, Fig. S1).

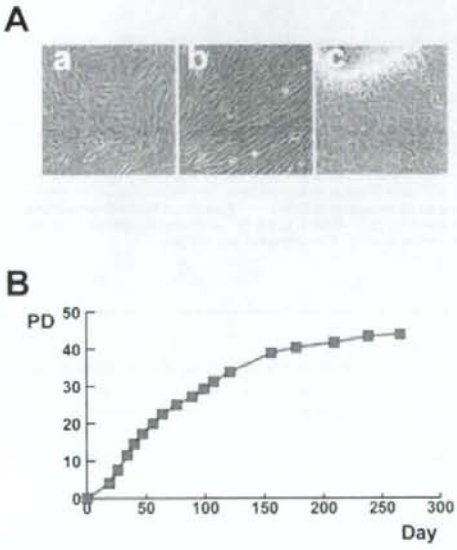

Figure 1. Proliferation of human 'sclera'-derived cells. A. Photograph of primary cultured human 'sclera'-derived cells by phase-contrast microscope. **B.** Growth curve of cultured human 'sclera'-derived cells. Vertical axis indicates population doublings (PD) and horizontal axis indicates days after innoculation of human 'sclera'-derived cells.
doi:10.1371/journal.pone.0003709.g001

Table 1. Human cells analyzed in this study.

Title	Description
Bone marrow	Bone marrow-derived cell (P1)
Hepatocyte	Hepatocyte (P0)
Endometrium	Endometrial cell
Synovium	Synovium-derived cell (P1)
Joint fluid	Joint fluid-derived cell (P1)
Muscle	Muscle-derived cell (P1)
Bone	Cancellous bone-derived cell (P1)
Fat	Subcutaneus fat-derived cell (P1)
Amniotic epithelium	Amniotic epithelial cell (P4)
Umbilical cord (1)	Umbilical cord-derived cell (P0) (1)
Umbilical cord (2)	Umbilical cord-derived cell (P0) (2)
Cartilage	Auricular cartilage-derived cell (P1)
Sclera	Sclera-derived cell (P1)
Cornea (stroma)	Keratocyte (P1)
Periostium	Periostium-derived cell (P1)
Dermis	Dermal fibroblast (P2)
Cortical bone	Cortical bone-derived cell (P3)

Gene chip analysis was performed using RNAs from the cells obtained from each tissue. The cells obtained from bone marrow, liver, synovium, joint fluid, muscle, bone, and fat were cultivated as previously described [31–33]. Amniotic epithelial cells and umbilical cord-derived cells were cultured after each tissue was manually separated from the placenta and minced by surgical knife and scissors. Auricular cartilage-derived cells, periostium-derived cells, dermal fibroblasts, and cortical bone-derived cells started to be cultured after each tissue was manually separated from surgical specimens from patients with polydactyly or microtia. Keratocytes and scleral cells were obtained from corneal stroma and sclera (also see the Materials and Methods section). "Endometrium" was obtained from the homogenized endometrial cells under liquid nitrogen. All cells were harvested under signed informed consent, with the approval of the Ethics Committee of the National Institute for Child and Health Development, Tokyo. Signed informed consent was obtained from donors and the surgical specimens were irreversibly de-identified. All experiments handling human cells and tissues were performed in line with the Tenets of the Declaration of Helsinki. Global gene expression profiles of those cells are uploaded to GEO accession #GSE10934 at http://www.ncbi.nlm.nih.gov/geo/index.cgi.
P: passage. P0 and P1 represents primary cell culture and cell culture one passage after starting primary culture from tissues, respectively.
doi:10.1371/journal.pone.0003709.t001

Chondrogenesis of human scleral cells

After reaching 70–80% sub-confluence, we started the micromass culture of scleral cells. Four weeks after culture in a chondrogenic medium containg TGF-β1 and BMP2, a pellet of human scleral cells exhibited a spherical shape (Fig. 3A). This pellet showed an alcian blue positive extracellular matrix, indicating that cultured micromasses of scleral cells exposed to TGF-β1 and BMP2 produce an abundant matrix (Fig. 3B). RT-PCR analysis demonstrated that scleral cells at passage 0 expressed aggrecan, COL2A, SOX5, SOX6, SOX9, and PTHR1 mRNAs

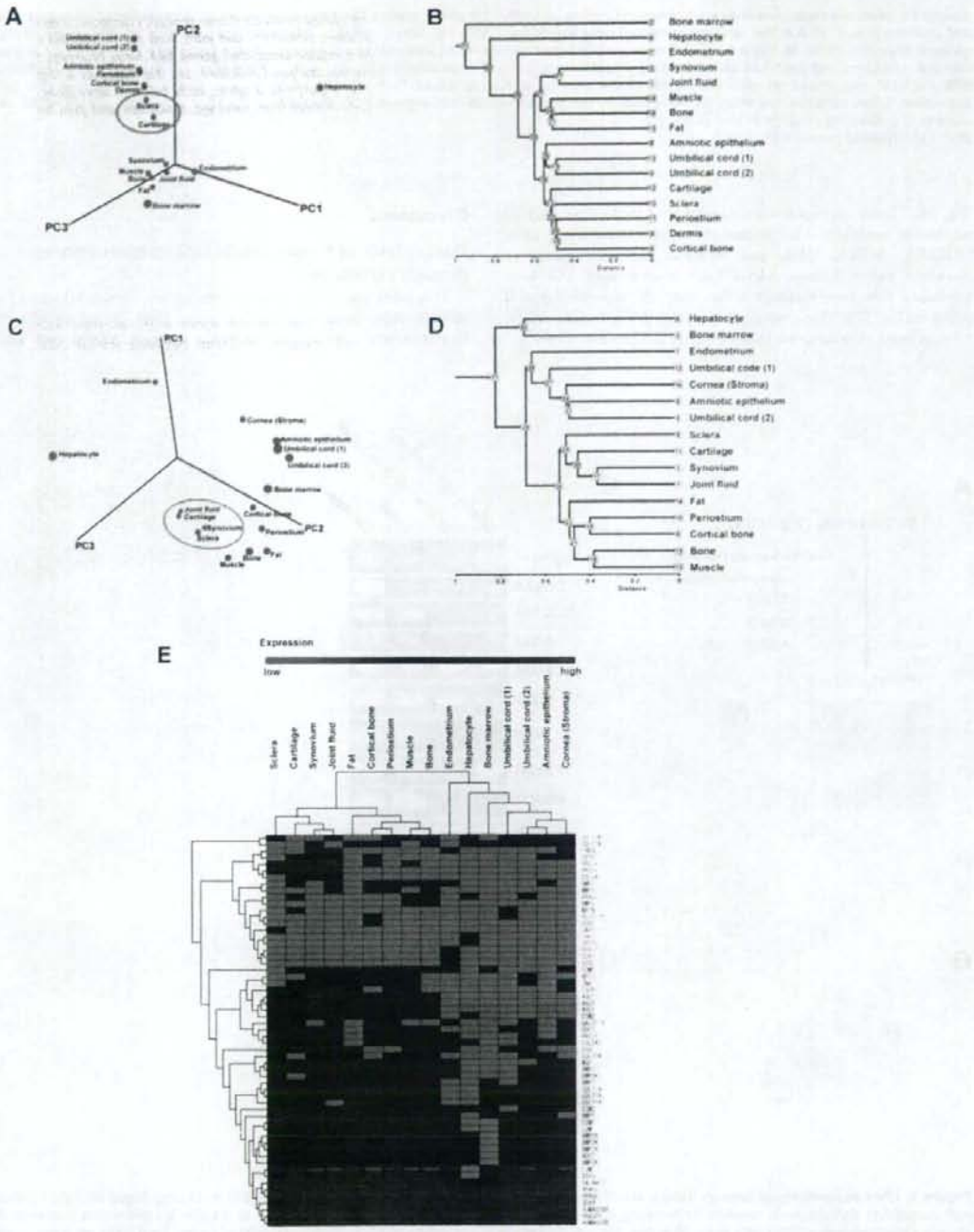

Figure 2. Global gene expression analysis of cultured human cells. A. Three-dimensional representation of PCA of gene expression levels (Human Genome U133 Plus 2.0: 54,675 probes). The gene expression data from scleral cells following one passage from the primary cultured cells (equivalent to appropriately 4 PDs) were used for PCA. Sclera and cartilage are positioned closely adjacent (shown in circle). **B**. Hierarchical clustering

analysis based on the expression of all genes (Human Genome U133 Plus 2.0: 54,675 probes, NIA Array Analysis) shows similarity between scleral cells and chondrocytes. **C**. PCA of the cartilage-associated gene expression (Table S1). Sclera, cartilage, synovium, and joint fluid are positioned closely adjacent (shown in circle). **D**. Hierarchical clustering analysis based on expression levels of the cartilage-associated genes (NIA Array Analysis). Sclera, cartilage, synovium, and joint fluid are categorized into the same group. **E**. Hierarchical clustering analysis (TIGR MeV, see the Materials & Methods) with the heat map, based on expression levels of the cartilage-associated genes. Each row represents a gene; each column represents a cell population. Sclera, cartilage, synovium, and joint fluid are categorized into the same group. Cells derived from cartilage, synovium, and joint fluid are capable of generating cartilage in vivo [7,34].
doi:10.1371/journal.pone.0003709.g002

(Fig. 3C). These expressions were maintained in the cells after 10 population doublings. After in vitro chondrogenesis of scleral cells, COL10A, SOX5, IHH, and MMP13 mRNA expressions increased. After human scleral cells labeled with DiI were implanted into a rat cartilage defect, the cells expressed type II collagen (Fig. 3D). These results demonstrated that human scleral cells retained chondrogenic potential both in vitro and in vivo.

Discussion

Tracing back of human scleral cells to chondrocytes through cultivation

This study was undertaken to investigate if human sclera has a chondrogenic nature like chicken sclera [8,9]. Bioinformatics of human scleral cells suggest similarity between scleral cells and

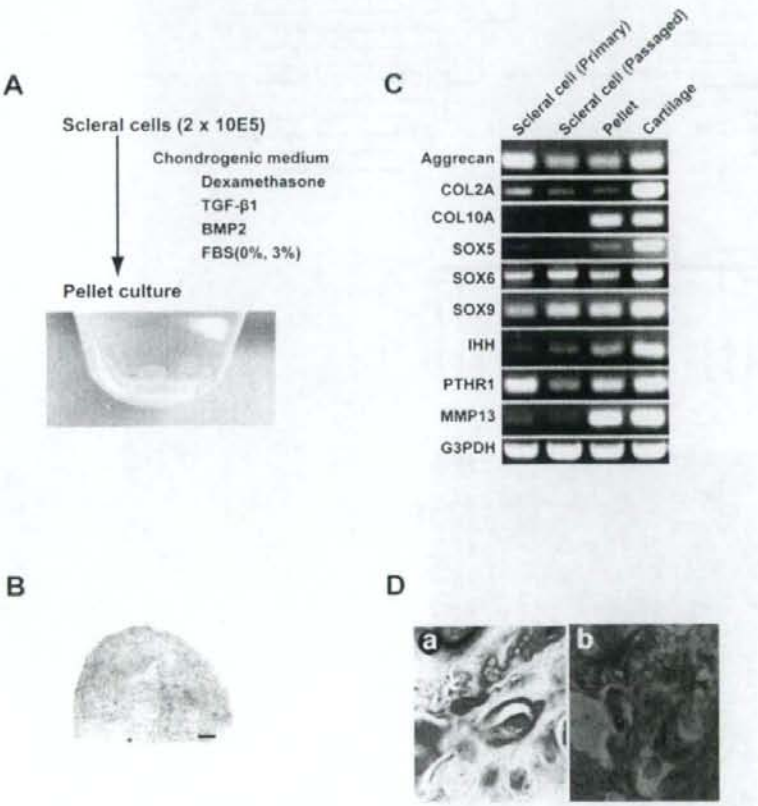

Figure 3. Chondrogenesis of human 'sclera'-derived cells. A. In vitro chondrogenesis. 'Sclera'-derived cells were centrifuged to make a pellet and cultured in chondrogenic medium for 4 weeks. Macroscopic feature is shown. **B**. Histological section of a pellet by micromass culture in a chondrogenic medium stained with alcian blue. Bar: 100 μm. **C**. Reverse transcriptase-PCR for cartilage-associated genes. Total RNAs were prepared from scleral cells at passage 0, at 10 population doublings, after in vitro chondrogenic induction, and normal cartilage as a positive control. **D**. Histological sections 4 weeks after transplantation of human scleral cells into cartilage defect of the knee in a rat. (a) Toluidin blue staining. (b) Immunohistochemistry. Human scleral cells were labeled with DiI (red). Nuclei were stained with DAPI (blue). Type II collagen was shown as green.
doi:10.1371/journal.pone.0003709.g003

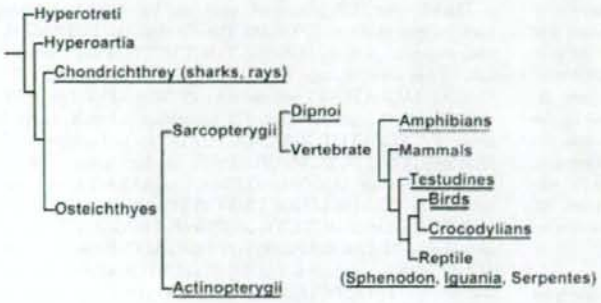

Figure 4

Figure 4. The distribution of scleral cartilage in vertebrates. The chondrogenic nature of the sclera is conserved across species. The figure is modified from Franz-Odendaal, TA, et al., 2006 [10]. Species that have cartilage in the sclera are underlined; species with either absence or presence of cartilage in the sclera, depending on family, are dot-underlined; species without cartilage in the sclera are non-underlined.
doi:10.1371/journal.pone.0003709.g004

chondrocytes, and this similarity may be attributed to evolution of the sclera (Fig. 4), that is, animals such as elasmobranch, teleost fish, amphibians, reptiles and birds incorporate the development of a cup of hyaline cartilage in the sclera [10]. Scleral cartilage is hypothesized to counter against the traction force of the extraocular muscle and against the accommodative force to move or deform the lens by intraocular muscles. In this paper, we employ the global gene expression approach to human scleral cells. As a result, scleral cells and chondrocytes are found to share common chondrogenic characteristics.

Simulation of chondrogenic process during development

The phenotype of the differentiated chondrocyte is characterized by the synthesis, deposition, and maintenance of cartilage-specific extracellular matrix molecules, including type II collagen and aggrecan [11–13]. Three-dimensional culture is a prerequisite for exhibition of this chondrogenic phenotype in vitro since the phenotype of differentiated chondrocytes is unstable in culture and is rapidly lost during serial monolayer subculturing [14–16]. The expression pattern of cartilage-associated genes in sclera-derived cells after induction is consistent with that of chondrocytes during development (Fig. 3C, Fig. S2): a) Consistent expression of type II collagen and aggrecan, markers of early-phase chondrogenesis [17,18] in sclera-derived cells, indicates that sclera-derived cells retain their chondrogenic nature as a default state; b) Induction of type X collagen and MMP13 genes after pellet formation of sclera-derived cells may simulate late-stage chondrogenesis. In addition, other chondrocyte-associated genes, such as sox5, IHH, and PTHR1 were also up-regulated. Sox5 functions as a transcription factor necessary for chondrogenesis [19,20], IHH promotes chondrogenesis as a cytokine [21], and PTHR1 mediates parathyroid hormone signaling as a specific receptor [18]. These results suggest that ex vivo culture of sclera-derived cells simulates the developmental process of chondrogenesis. Despite the chondrogenic nature of sclera-derived cells, lack of cartilage in the sclera in humans may be attributed to cis- and trans-regulation of cartilage-associated gene(s), or an unclarified inhibitory mechanism that was altered during evolution (Fig. 4).

Implication of chondrogenic nature of sclera in diseases

The fact that the gene expression pattern of the human fibrous sclera is similar to that of cartilage is interesting not only as comparative anatomy but also from a patho-etiological view point. The sclera and the joint cartilage are common targets for inflammatory cells in rheumatic arthritis [22,23] or polychondritis [24], implying common proteins between the sclera and the synovium. Although the target protein(s) remains unclarified, our findings directly explain an enigma that both the sclera and the joint cartilage are affected in rheumatic arthritis. Furthermore, mutations in genes for type II and type XI collagen are a cause of Stickler syndrome [25,26]. Patients with Stickler syndrome have joint deformity and severe high myopia due to an abnormality of the sclera. These affected lesions may be attributed to the chondrogenic nature of human sclera. In conclusion, our present study shows a chondrogenic potential of human sclera and explains the etiology of scleral disorders, at least in part. In addition, we would like to emphasize that the first database of gene expression in the human infant sclera (uploaded to GEO accession #GSE10934 at http://www.ncbi.nlm.nih.gov/geo/index.cgi) may contribute to the elucidation of scleral diseases in the future.

Materials and Methods

Isolation and cell culture of human scleral cells

Scleral tissues were excised from surgical specimens as a therapy of retinoblastoma, under signed informed consent, with the approval (approval number, #156) of the Ethics Committee of the National Institute for Child and Health Development, Tokyo. Signed informed consent was obtained from donors, and the surgical specimens were irreversibly de-identified. All experiments handling human cells and tissues were performed in line with the Tenets of the Declaration of Helsinki. The scleral pieces were cut into smaller pieces and cultured in the growth medium (GM): Dulbecco's modified Eagle's medium (DMEM)/Nutrient mixture F12 (1:1) with high glucose supplemented with 10% fetal bovine serum, insulin-transferrin-selenium, and MEM-NEAA (GIBCO).

Oligonucleotide microarray

Total RNAs were isolated from cultured scleral cells in the growth medium without any induction of differentiation to perform the gene chip analysis. Total RNA was extracted from a total of 5×10^6 cultured human scleral cells and other mesenchymal cells (Table 1) using RNeasy Plus mini-kit® (Qiagen, Maryland, USA) according to

the manufacturer's instructions. A comprehensive expression analysis was performed using 2 μg of total RNA from each sample and GeneChip® Human Genome U133 plus 2.0 probe arrays (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. To normalize the variations in staining intensity among chips, the 'Signal' values for all probes on a given chip were divided by the median value for expression of all genes on the chip. To consider genes containing only a background signal, probes were eliminated only if the 'Signal' value was less than 10, or the Detection call was 'Absent' in any sample using GeneSpring software version 7.2 (Agilent Technologies, Palo Alto). The gene chip analysis was carried out on 8 independent scleral cultures.

Hierarchical clustering and principal component analysis (PCA)

To analyze the gene expression data in an unsupervised manner by gene chip array, we used hierarchical clustering and principal component analysis (NIA Array; http://lgsun.grc.nia.nih.gov/ANOVA/ [27], TIGR MeV; http://www.tm4.org/mer.html [28]). The hierarchical clustering techniques classify data by similarity and the results are represented by dendrogram. PCA is a multivariate analysis technique which finds major patterns in data variability. Hierarchical clustering and PCA were performed on the data of gene chip analysis (a single assay for each sample) to group scleral cells and other mesenchymal cells into subcategories (Table 1).

In vitro chondrogenesis

Two hundred thousand scleral cells were placed in a 15-ml polypropylene tube (Becton Dickinson) and centrifuged for 10 minutes. The pellet was cultured in DF-C medium™ containing 0.1 μM dexamethasone, 1 mM sodium pyruvate, 0.17 mM ascorbic acid-2-phosphate, 0.35 mM proline, 6.25 μg/ml bovine insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenous acid, 5.33 μg/ml linoleic acid, 1.25 mg/ml BSA, 5 ng/ml TGF-β1, 5 ng/ml BMP2, and 3% fetal bovine serum (TOYOBO). The medium was replaced every 3 to 4 days for 28 days. For microscopy, the pellets were embedded in paraffin, cut into 5-μm sections, and stained with alcian blue [29,30].

In vivo chondrogenesis

Under anesthesia, full thickness cartilage defects were created in the trochlear groove of the femur in SD rats. The defects were filled with DiI-labeled human scleral cells. The rats were returned to their cages after the operation and allowed to move freely. Animals were sacrificed with an overdose of sodium pentobarbital at 4 weeks after the operation. Specimens were dissected and embedded in paraffin. The sections were stained with toluidine blue and immunohistochemically stained with anti-type II collagen antibodies (clone F-57, DAIICHI FINE CHEMICAL, Co. Ltd., Toyama, Japan). All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the US National Institutes of Health (NIH Publication No. 86-23, revised 1985). The operation protocols were accepted by the Laboratory Animal Care and Use Committee of the Research Institute for Child and Health Development (2003-002).

Reverse transcriptase-PCR

Total RNA was isolated with an RNeasy Plus mini-kit. Cartilage pellets were digested with 3 mg/ml Collagenase D for 3 hours at 37°C.

The following PCR primer sets were used for cartilage-associated genes: aggrecan, sense (5'-TACACTGGCGAGCACTGTAAC -3') and antisense (5'-CAGTGGCCCTGGTACTTGTT-3'), product size, 71 bp; collagen, type II, alpha 1, sense (5'-TTCAGCTATGGAGATGACAATC -3') and antisense (5'-AGAGTCCTAGAGTGACTGAG -3'), product size, 472 bp; collagen, typeX, alpha 1, sense (5'-CACCTTCTGCACTGCTCATC-3') and antisense (5'-GGCAGCATATTCTCAGATGGA-3'), product sizem, 104 bp; SOX5, sense (5'-AGCCAGAGTTAGCACAATAGG -3') and antisense (5'-CATGATTGCCTTGTATTC -3'), product size, 619 bp; SOX6, sense (5'-ACTGTGGCTGAAGCACGAGTC -3') and antisense (5'-TCCGCCATCTGTCTTCATACC -3'), product size, 562 bp; SOX9, sense (5'-GTACCCGCACTTGCACAAC-3') and antisense (5'-TCGCTCTCGTTCAGAAGTCTC-3'), product size 72 bp; Indian hedgehog homolog (IHH), sense (5'-TGCATTGCTCCGTCAAGTC-3') and antisense (5'-CCACTCTCCAGGCGTACCT-3'), product size 88 bp; parathyroid hormone receptor 1(PTHR1), sense (5'-CCTGAGTCTGAGGAGGACAAG-3') and antisense (5'-CACAGGATGTGGTCCCATT-3'), product size 86 bp; matrix metallopeptidase 13 (MMP13), sense (5'-CCAGTCTCCGAGGAGAAACA-3') and antisense (5'-AAAAACAGCTCCGCATCAAC-3'),product size, 85 bp, and GAPDH, sense (5'-GCTCAGACACCATGGGGAAGGT-3') and antisense (5'-GTGGTGCAGGAGGCATTGCTGA-3'), product size, 474 bp.

Supporting Information

Figure S1 Global gene expression analysis of cultured human cells. Hierarchical clustering analysis based on expression levels of the cartilage-associated genes (NIA Array Analysis). We performed gene chip analysis (a single assay for each analysis) for eight independent primary scleral cultures from five patients (donors). We started eight independent cultures from three different scleral sites of Donor 2 (e.g. the anterior site 1.5 mm apart from the limbs, the middle part, and the posterior part), 2 different scleral sites of Donor 5, and three scleral sites of Donor 1, 3, and 4. We performed hierarchical clustering analysis, using these independent cultures and obtained consistent results, that is, "sclera"-derived cells are categorized into one sub-group. Furthermore, the sclera, cartilage, synovium, and joint fluid are categorized into the same group.

Found at: doi:10.1371/journal.pone.0003709.s001 (0.07 MB PDF)

Figure S2 Cartilage-associated gene expressions in cultured fibroblasts derived from the dermis and the sclera. Cartilage-associated gene expressions by RT-PCR in cultured fibroblasts derived from the dermis and the sclera. Aggrecan, COL2A, IHH and PTHR mRNA expressions were clearly stronger in the scleral fibroblasts compared to the dermal fibroblasts, indicating that chondrogenic nature could be specific for the sclera among collagenous tissues.

Found at: doi:10.1371/journal.pone.0003709.s002 (0.01 MB PDF)

Table S1 Cartilage-associated genes

Found at: doi:10.1371/journal.pone.0003709.s003 (0.01 MB PDF)

Acknowledgments

We would like to express our sincere thanks to T. Tang for helping with animal experiments and T. Sugiki, M. Nasu, A. Kawakita and M. Toyoda for their discussion of this work.

Author Contributions

Conceived and designed the experiments: YS NA TM IS AU. Performed the experiments: YS NA HM TM IS. Analyzed the data: YS YT KM HS IS. Contributed reagents/materials/analysis tools: YS NA IS. Wrote the paper: YS KM HS IS AU.

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Symposium: Nuclear reprogramming and the control of differentiation in mammalian embryos

Elucidating nuclear reprogramming mechanisms: taking a synergistic approach

Dr Hidenori Akutsu became interested in nuclear reprogramming in mammalian species when he was a research fellow at University of Hawaii under Dr Ryuzo Yanagimachi. This interest endured and motivated him to undertake further research under Dr Minoru Ko at NIA/NIH (embryo genomics) and Dr Kevin Eggan at Harvard University (epigenetic and nuclear reprogramming). While at Harvard University he also became an important part of Dr Douglas Melton's team, deriving human embryonic stem cell lines which were later offered freely to the scientific community to facilitate the efforts of other scientists. His special interests are egg development, epigenetic and nuclear reprogramming and embryonic stem cells.

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Abstract

Nuclear reprogramming is the process by which a differentiated somatic nucleus has developmental potential restored to it. It involves heritable changes in gene expression as well as structural and functional changes to chromatin structure. This process is naturally induced immediately after fertilization, but can also be artificially induced by nuclear transfer, cell fusion and also now by viral transduction with four stem cell genes. However, the frequency of successful reprogramming is low in each system. The highest success rates, those using nuclear transfer, are only of the order of 2–5%. This article briefly reviews these three methods and proposes a synergistic approach where conditions that facilitate reprogramming in one system are transposed to the others. This might increase the incidence of successful reprogramming and identify common steps necessary for the reacquisition of developmental potential.

Keywords: *developmental potential, differentiation, embryonic stem cell, nuclear reprogramming, nuclear transfer, pluripotency*

Cell differentiation and nuclear reprogramming

Cell differentiation is the process by which a cell becomes specialised to perform specific biological functions (Gurdon, 1968). The process is associated with a decline in the range of cell types that the cell is capable of generating (Gurdon, 1968). It had been initially thought that as cells differentiated, hereditary material no longer required was cast off or permanently inactivated (Weismann, 1893). However, this paradigm was shown to be false more than 50 years ago when Briggs and King transferred differentiated nuclei from blastula cells to enucleated eggs of the frog *Rana pipiens* (Briggs and King, 1952). These reconstructed cells went on to generate normal hatched embryos, showing that nuclei of differentiated cells contain the same genetic material as those of undifferentiated cells. The current paradigm for how cell differentiation occurs involves the assembly of condensed chromosomal structures (Kass and Wolffe, 1998). Such structures, formed via interactions between DNA and protein, are thought to compartmentalize chromatin into functional domains and, in some unknown way, stably maintain the differentiated state even when the cell divides.

In terms of mammalian development, differentiation first occurs at the blastocyst stage in the preimplantation embryo. As the embryo develops, the outer layer cells of the embryo (the trophectoderm) become morphologically distinct from the inner cell mass (ICM). Cells of the trophectoderm and ICM have different developmental potentials, e.g. cells of the ICM have the potential to form all the cells of the conceptus, whereas the trophectoderm cells have only the potential to form extraembryonic cells or the placenta.

The processes responsible for the epigenetic changes that lead to dedifferentiation are referred to as nuclear reprogramming mechanisms (Rideout *et al.*, 2001). Nuclear reprogramming in this sense refers to the process by which a specialized nucleus re-acquires developmental capacity. This definition includes complete reprogramming to a totipotent state (verifiable only by generation of viable offspring) and also partial reprogramming where pluripotency (the capacity to generate cells representative of all three germ layers) is restored. By necessity, it involves heritable changes to gene expression, i.e. changes in gene expression that are passed on to daughter cells. Some have suggested that transient changes to gene expression constitute nuclear reprogramming (Hakelien *et al.*, 2002), but such changes do not persist, nor is there any evidence that they are transferred to progeny cells. Such observations almost certainly result from residual transcription activity rather than the consequence of a reprogrammed genome, and so these examples do not constitute nuclear reprogramming as defined here and elsewhere (Hochedlinger and Jaenisch, 2006).

Naturally induced nuclear reprogramming

The differentiated state of cells is found to be extremely stable (Kato and Gurdon, 1993). The only stage during which normal mammalian cells seem to naturally dedifferentiate immediately follows fertilization (Schultz *et al.*, 1999). The sperm and oocyte, both highly differentiated cells with condensed chromatin structure, fuse to produce a zygote. Within the zygote, changes lead to the reversion to a less specialised totipotent cellular state (Kelly, 1977). Although the mechanism responsible is unknown, two events are associated with this dedifferentiation: chromatin structure becomes less dense: protamines are removed from sperm-derived chromatin and replaced by oocyte-derived histones (Perreault, 1992); and methylated haploid parental genomes are demethylated (Barton *et al.*, 2001).

Additionally, it has been speculated that inappropriate or incomplete nuclear reprogramming may occur in a pathological context, i.e. during the generation of teratomas. Teratomas are benign tumours associated with chaotic cell-lineage formation. The 'dedifferentiation' theory of cancer states that such lineages may arise from cells that have undergone dedifferentiation to a multipotent state (Ribbert, 1911). Teratomas can also be produced experimentally by injection of pluripotent stem cells into ectopic sites of a syngeneic animal (Evans and Kaufman, 1981; Matsui *et al.*, 1992; Rensnick *et al.*, 1992), so it is conceivable that inappropriately reprogrammed somatic cells could be the origin of such cancers.

Artificially induced nuclear reprogramming

In non-transformed somatic cells, once the differentiation programme of a cell has started, the process is normally irreversible. However, this programme may be reversed artificially. Using nuclear transfer (NT) (Wilmut *et al.*, 1997), cell fusion (Tada *et al.*, 1997), or even viral transduction of four specific stem cell genes (Takahashi and Yamanaka, 2006), it is possible to artificially and heritably alter a cell's gene expression and its functional identity. These techniques are collectively termed 'artificial induction of nuclear reprogramming' (**Figure 1**). The conversion of differentiated cells to pluripotent cells illustrates that cells do not permanently lose the ability to be pluripotent during differentiation.

Frustratingly, the mechanism by which a somatic nucleus may be reprogrammed remains unknown, aside from the fact that such a mechanism almost certainly involves both structural (Kikyo *et al.*, 2000) and chemical (Monk *et al.*, 1987) changes to chromatin. It may be possible for human somatic cells to be reprogrammed to a pluripotent state. If successful, this strategy would provide a potentially endless source of cells for biological research, as well as medical applications (Stojkovic *et al.*, 2005; Verlinsky *et al.*, 2005), toxicity assessment, drug testing and possibly even gene therapy (Wobus and Boheler, 2005). **Figure 2** illustrates how identification of reprogramming molecules and mechanisms could facilitate cell replacement therapy in humans. Over the past century, organ transplantation has evolved rapidly to the current widespread use of donated organs for the treatment of end-stage kidney, heart, and liver failure. However, with limited supplies of organs and an increasing demand for them, many patients who need transplants do not receive them (Gridelli and Remuzzi, 2000). The increasing gap between supply and demand for tissue and organ transplants means that harnessing nuclear reprogramming mechanisms is important (Sullivan and Eggan, 2007).

Nuclear transfer: the oldest and still the most reliable reprogramming technique

Spemann (1938) originally suggested transplantation of nuclei between cells as a technique to study the role of genetic material in cellular differentiation. In nuclear transfer, the nucleus from a differentiated donor cell is transplanted into an enucleated oocyte. The oocyte can reprogramme even an adult differentiated nucleus and the new cell can develop as an embryo. Artificially induced nuclear reprogramming by NT was first demonstrated by Briggs and King in 1952, when they showed that transfer of somatic nuclei to enucleated eggs can direct development so that tadpoles are generated (Briggs and King, 1952). Gurdon later refined the technique so that adult and fertile frogs could be generated (Gurdon *et al.* 1958). Decades later, the production of 'Dolly', the first viable mammal derived by reprogramming a fully differentiated adult somatic cell, illustrated that the mammalian nuclear genome can be completely reprogrammed and totipotency of the nucleus restored (Wilmut *et al.*, 1997). The nuclei of these offspring contain genomes of identical sequence to that of the nuclear donor. At present, nuclear transfer is the only technique in which one can accomplish total nuclear reprogramming in an unequivocal manner; by deriving viable offspring from a reconstituted embryo. More recently, embryonic stem (ES) cells have been derived from cloned mice (Wakayama *et al.*, 2001). The ES cells produced by somatic cell nuclear transfer retained self-renewal and pluripotent features, contributing to all germ layers, including the germline. In addition, gene expression profiling experiments showed the ES cell lines derived from cloned and fertilized mouse blastocysts are indistinguishable (Brambrink *et al.*, 2006). The NT-ES cells are developmentally and functionally equivalent to the fertilization-derived ES cells.

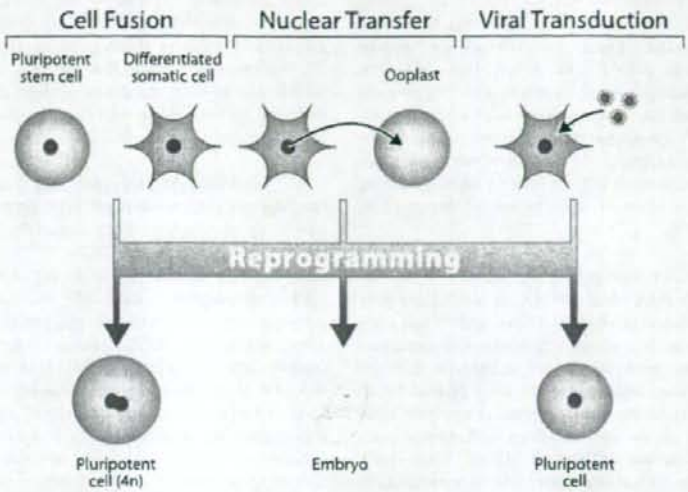

Figure 1. Artificially induced nuclear reprogramming. Cell fusion: a somatic cell fused with a pluripotent stem cell can be reprogrammed in the hybrid cell. These fused hybrid cells show similar features as embryonic stem (ES) cells; however the hybrid cell has a tetraploid karyotype and is unable to contribute to chimeras. Nuclear transfer: an adult somatic cell is transferred into an enucleated oocyte followed by artificial activation. These nuclear-transferred embryos can produce ES cells which are pluripotent, contributing to all germ layers including the germ cell lineage. Viral transduction: a somatic cell transduced by retroviruses expressing four key genes, *Oct3/4, Sox2, Klf4* and *c-Myc*, can be reprogrammed into iPS cells resembling ES cells in a cell-autonomous fashion. Only the nuclear transfer method can produce viable animals as it can return an adult nucleus to a totipotent, embryonic state.

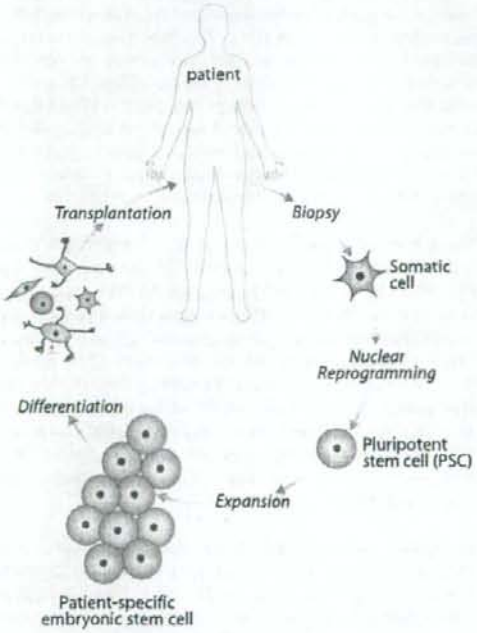

Figure 2. The ultimate goal of nuclear reprogramming research: controlled restoration of developmental potential. Once the mechanism by which nuclear reprogramming is understood, human somatic cells could be induced to dedifferentiate into pluripotent stem cells (PSC). PSC could then be expanded in culture and induced to redifferentiate into the cell type(s) required by the patient. These non-allogenic differentiated cells could then be transplanted into the patient with a decreased risk of immunorejection. It is also important to point out that patient matched pluripotent stem cells can also serve as in-vitro models for studying human disease and development at a cellular and molecular level. Such reprogramming will also allow the generation of genetically matched ES cells will, in themselves, provide scientists and clinicians an important new tool to recapitulate onset of specific diseases *in vitro* (Di Giorgio *et al.*, 2007).

Successful reprogramming of somatic nuclei by placing them in enucleated oocytes should perhaps not have been completely unexpected. There are compelling reasons why a system should exist for the removal of epigenetic modifications (excluding gametic imprints) in the oocytes and sperm. They are both highly specialized differentiated cells, and removal of their epigenetic patterns is essential to allow development of pluripotent cells from the inner cell mass (ICM). The same mechanism may be causing reprogramming of a somatic nucleus when exposed to the cytoplasm of an oocyte (Surani, 1999).

Many variables affect reprogramming success with NT. Some of these have been identified, i.e. structural integrity of the nuclear membrane (Willadsen 1986), quality and copy number of donor genetic material, chromatin conformation, histone composition, methylation and acetylation patterns (Campbell, 1999). Also important is the level of maturation or mitosis promoting factor (MPF) (Fulka *et al.*, 1996) and synchronization of donor and recipient cell cycles prior to embryo reconstruction (Campbell, 1996). High MPF concentrations in the oocyte and appropriate synchronization of donor and nuclear cell cycle using serum starvation are thought to minimize chromosomal damage and promote generation of reconstructed embryos that divide to produce normal diploid daughter cells.

Campbell suggests that the frequency of live offspring generation from reconstructed mammalian embryos made by NT is improved when the donor nuclei are in a quiescent state (Campbell *et al.*, 1996; Campbell, 1999). The successful production of Dolly, the first viable animal to be generated by nuclear transfer, used a nucleus from a cultured adult-differentiated somatic cell that had been serum starved into quiescence (Wilmut *et al.*, 1997). Kato *et al.* (1998) reported cloning of calves at 80% success ratio based on the number of transferred embryos using quiescent cumulus cells and oviduct epithelial cells that were cultured for several passages followed by serum starvation. Alternatively, using non-cultured cells also succeeded in producing cloned animals. Wakayama *et al.* (1998) used mouse cumulus cells, a naturally quiescent cell population, as nuclear donating cells in successful nuclear transfer experiments with mouse ooplasts. Ogura *et al.* (2000) made cloned mice by transferring Sertoli cells into enucleated mature oocytes. In both of these experiments, the cell cycle stage of the nuclear donors was controlled but the possibility that animals can be generated using non-quiescent cells as nuclear donors cannot be dismissed. Other researchers claim successful generation of mammalian offspring from nuclei not intentionally induced into a quiescent state (Cibelli *et al.*, 1998). Also, the possibility that transferred nuclei in Wakayama's and Ogura's experiments were non-quiescent cannot be eliminated.

Presumably, the importance of the state of the donor nucleus cell cycle is directly linked to compatibility with the recipient oocyte cytoplasm. Metaphase of second meiotic division (MII) oocytes has typically become the state of choice of recipient cytoplasts for NT procedures (Campbell *et al.*, 1996). MII oocytes contain active MPF to induce nuclear envelope breakdown (NEBD), premature chromosome condensation (PCC), and dispersion of nucleoli in the transferred nucleus, which may be essential for nuclear reprogramming. The donor nucleus in S phase of the cell cycle is likely to be incompatible with a high MPF state, leading to DNA damage and arrest at an early cleavage stage. However, inter-species NT experiments suggest that the occurrence and extent of NEBD and PCC in the donor nucleus are variable between different species, donor cell types and different procedures (Meissner and Jaenisch, 2006).

It had been thought previously that only the cytoplasm of the MII oocyte can support reprogramming after NT, so numerous species have been cloned by NT into MII oocyte (Meissner and Jaenisch, 2006). It seemed necessary for initiating reprogramming that the donor nucleus had elevated MPF concentrations, since NT embryos fail to develop, transforming into interphase zygotes (McGrath and Solter, 1984; Wakayama, 2000). However, more recently, a new insight disproving a myth of MII necessity for NT has been reported (Egli *et al.*, 2007). Unlike interphase zygotes, fertilized zygotes arrested in mitosis can fully support the reprogramming of somatic cells to the totipotent state. This indicates that factors sufficient for reprogramming are not limited to oocytes, and suggests that a continuum of activity extends beyond the unfertilized egg (Egli *et al.*, 2007). Why is the metaphase cell useful for reprogramming? A possible explanation is that condensed chromatin expels transcription factors like Oct-3/4 and Sox2 (Martinez-Balbas *et al.*, 1995), and without a nuclear membrane to enclose them, they are free to interact with any foreign chromatin introduced. Also, as the cell is poised to divide in M phase, it has synthesized many components of the cell to elevated levels, so presumably factors necessary for reprogramming are present in a greater abundance than at other stages of the cell cycle.

Experiments by Eggan *et al.* (2001) show that the number of live mice generated from cells reprogrammed via nuclear transfer is dependent on the genetics of the mouse from which the nuclear donor cell is taken. ES cells taken from inbred 129/SvJae mice fail to produce any post-natal surviving offspring, whereas cloned pups derived from ES cells of C57BL/6 and 129/SvJae matings can survive to adulthood. It may be that the use of inbred animals as nuclear donors introduces a reprogramming barrier not present in hybrids strains. Investigating why this occurs might elucidate more about mechanisms involved in nuclear reprogramming.

Much remains to be learnt about how somatic nuclei are reprogrammed after being transferred into ooplasts. For example, what factors and signalling pathways are involved in altering the chromatin structure, methylation patterns, and gene expression during reprogramming? Is there a master trigger that induces a cascade of downstream events or does it take several factors working in parallel pathways to initiate reprogramming? This might be the case as the frequency of successful reprogramming is so low. How do subtle epigenetic differences from normal animals, such as methylation/acetylation patterns, contribute to the abnormalities that cloned animals often exhibit?

In summary, successful production of cloned animals by NT proved that somatic nuclei could reverse their developmental clock to recreate totipotency in the oocyte. The transferred nuclei must be reprogrammed in resetting of an embryonic transcriptional programme. Although NT remains the tool

of choice for studying reprogramming at a functional level, less technically demanding approaches may be helpful for dissecting reprogramming at the cellular, molecular and biological levels (Hochedlinger and Jaenisch, 2006).

Cell fusion: a reprogramming system with the challenge of tetraploidy

Cell fusion is the mechanism by which reprogramming occurs naturally: a haploid oocyte fuses with a haploid spermatozoan. Artificially induced cell fusion generates tetraploid cells which, due to their lack of contribution to chimeras and their perceived susceptibility to turn aneuploid and abnormal, are of limited therapeutic use (Tada *et al.*, 1997; Sullivan and Eggan, 2007). However, cell fusion is the only system yet to show reprogramming in humans (Cowan *et al.*, 2005), and if it was possible to harness cell enucleation strategies either by naturally occurring (erythrocyte enucleation or selective genome ejection systems seen in insects species such as fire-ants) or artificial means (cytoplast/whole cell fusions, or manual chromatin removal), this problem could be surmounted (Sullivan and Eggan, 2007).

Cell fusion, apart from being a potential therapy, has provided a model system where aspects of how cell-specific phenotypes are initiated and maintained can be examined in fusion products of different cell types (intertypic synkaryons). Monoclonal antibodies and polymorphisms between fusion partners can be used to study gene expression at the single cell level or in mass cultures at a biochemical and molecular level. Regulatory mechanisms governing cell fate and differentiation have been partially elucidated by studying differences among cell types in the frequency, kinetics, and patterns of gene expression. The results of both strategies applied to heterokaryons and cell hybrids show that the expression of genes in the nuclei of differentiated cells is remarkably plastic and susceptible to modulation by the cytoplasm (Boshart *et al.*, 1993). Isolation of genetically stable cell hybrids can be achieved using selection for transgenes integrated in, or against mutations occurring in, only one of the parental cell types. Generation of cell hybrids has elucidated three principles of cell differentiation (Boshart *et al.*, 1993): (i) trans-acting gene regulators are involved in cell differentiation; (ii) such regulators repress as well as activate cell-specific gene expression; and (iii) maintenance of the differentiated state is dependent on such factors.

In intertypic somatic hybrids, genes associated with specialized function are often shut down. Such repression is termed 'extinction'. Extinction is a commonly observed feature of intertypic hybridization (Davidson, 1974). One interesting example of hybridization provided the first direct evidence that telomere length determines proliferative capacity in human cells (Wright *et al.*, 1996). In immortal cell lines, the ends of the chromosomes (telomeres) are constitutively replenished by the ribonucleoprotein enzyme telomerase (Counter *et al.*, 1992), while in somatic cell types, telomere length is found to shorten with age (Lindsey *et al.*, 1991; Vaziri, 1997). Hybrids of immortal and somatic cells are found to have limited life span, and this is due to the extinction of the telomerase gene (Wright, 1996). Treating these cell hybrids with specific oligonucleotides results in telomere elongation. It is thought that telomere elongation reduces the probability of DNases cutting into essential regulatory and expressed sequences in chromosomal DNA and so extends the life span of the hybrids (Wright *et al.*, 1996).

Gene repression is far more commonly observed than activation (Baron *et al.*, 1996). However, it has been observed that activation of cell-type specific gene expression can also occur when different cell types are fused (Baron *et al.*, 1996). An interesting example of activation involves fusing erythroid cells at different developmental stages (Broyles, 1999). The phenotype of hybrid cells involves the retention of specific chromosomes (Weiss and Chaplain, 1971), and is dependent on the number of copies of the individual chromosomes retained. For example in hepatoma × fibroblast hybrids possessing only one copy of hepatic chromosomes, the hepatic phenotype is not observed; if, however, the hybrid contains two sets of hepatic chromosomes, the hepatic phenotype is present. Clearly a delicate equilibrium between positive and negative trans-acting factors mediates hybrid phenotype (Peterson and Wess, 1972). It is interesting to juxtapose these data with similar findings from imprinting experiments injecting transgenes containing differentially methylated regions (Reik *et al.*, 1999). Introduction of such genes alters the methylation status of the chromosomal DNA, also indicating a trans-acting mechanism with a delicate equilibrium (Reik *et al.*, 1999).

In summary, cell hybridization experiments have shown that trans-acting gene regulators control the differentiated state of a cell. Somatic cells may be reprogrammed by fusion with pluripotent stem cells; however, in this case, the persistence of ES cell-derived chromatin causes applicative and interpretive complications, i.e. the resulting tetraploid cells are of limited therapeutic use and it is still unknown whether the ES cell chromatin remaining in the fusion product is playing an active role in the perpetuation of the resultant phenotype.

iPS cell transduction: a technique to study reprogramming at the molecular level

There is currently much interest in the reprogramming community surrounding 'induced pluripotent stem (iPS) cell transduction' (Takahashi and Yamanaka, 2006) (**Figure 1**), a novel approach that uses four transcription factors to restore an ES cell-like phenotype to murine fibroblasts (Rodolfa and Eggan, 2006). By simply transducing murine fibroblast cultures with Moloney virus coding for four stem cell factors (Oct3/4, Sox2, Klf4 and c-Myc), it appears that a pluripotent stem cell-like state can be restored. This is particularly exciting when one considers that the techniques involved (cell culture and viral transduction) are commonly used in many laboratories worldwide already. New work on iPS cells has recently been published from three different laboratories (Rodolfa *et al.* 2007). They showed iPS cells selected for Nanog expression can contribute to all tissue types including germ cells. Amazingly, the Nanog–iPS cells closely resemble ES cells in their epigenetic state as well as genetic activity (Okita *et al.*, 2007; Wernig *et al.*, 2007; Maherali *et al.*, 2007). Many laboratories worldwide can now use this method to elucidate

reprogramming mechanisms. Further published work with this technique is eagerly anticipated, as several questions have still to be answered: for example what cells are being transduced to generate these iPS cells? Can this be done with human cells? What is the molecular basis of reprogramming induced by the four factors? Is it the same process that happens during NT and cell fusion reprogramming? Can the implicated genes be activated and induce reprogramming without use of oncogenic virus (Surani, 2007)?

Screening for reprogramming factors

Reprogramming remains largely phenomenological, and efforts should now aim to dissect the mechanism at the molecular level (Hochedlinger and Jaenisch, 2006). Oocytes, preimplantation embryos, and pluripotent stem cells contain factors sufficient for reprogramming, and so constitute good material for identifying reprogramming factors (Hamatani *et al.*, 2004; Ko, 2006). Beyhan *et al.* (2007) reported global gene expression analysis of bovine NT, IVF embryos and donor somatic cells to characterize differences in their transcription profiles. They have found a small set of genes differentially expressed as well as genes of donor cells persistently expressed in NT embryos. Investigating gene expression changes that occur during or soon after reprogramming should elucidate the molecular mechanisms involved.

Another approach includes the use of mass spectrometry to identify reprogramming factors in cells and cell-derived extracts (Koziol *et al.*, 2007). Cell extracts have been shown to induce transient changes in gene expression and chromatin structure in differentiated cells (Dimitrov and Wolffe, 1996), which, if maintained, could possibly result in reprogramming. However, a caveat to these approaches is that the initial induction of reprogramming may only involve subtle changes in gene expression that then cumulatively elicit a pronounced effect. A more forceful approach would be to individually overexpress the four factors shown by Yamanaka and colleagues to reprogram differentiated cells (Takahashi and Yamanaka, 2006) and analyse the resulting genome-wide changes in gene expression. Alternatively, small molecule or RNAi screens could be performed to identify the important factors (Edwards, 2006).

Induction and maintenance of nuclear programmes has, for many years, been considered to be directed solely by proteins involved in gene regulation and morphogenic signalling. Many researchers have carried out reprogramming screens for proteins only to pull out generic chromatin remodeling factors. Additional candidates now need to be considered, including non-proteinaceous macromolecules. RNA, for example, has now emerged as a key player in a surprisingly large number of gene regulation studies. For example, the activity of X chromosomes in female mammals is controlled by non-coding RNAs such as *Xist* and *Tsix*. Furthermore, microRNAs (miRNAs), a large family of short non-coding RNAs (17–25 nucleotides) that mainly function to repress expression of their target genes, regulate blood development (Yekta *et al.* 2004). Tang *et al.* (2007) have recently showed a large proportion of the maternal genes are directly or indirectly under the control of miRNAs, which demonstrates that the maternal miRNAs are essential for the earliest stages of mouse embryonic development. It would not be surprising if non-coding RNA has further roles in specific and stable regulation of developmental programmes. miRNA may have an important role in nuclear reprogramming.

An alternative approach to studying artificial reprogramming, which could be expanded further, has been to study naturally induced reprogramming in lower vertebrates where it occurs successfully and more frequently and to look for common elements in more complex organisms. Unlike mammals, many fish and amphibia have the capacity to regenerate complex structures such as limbs after injury. Even mammals have this capacity in Msx1 expressing regions at the digit termini and more widely during early embryonic phases (Han *et al.*, 2003). This process involves cell migration and a change in cell phenotype in response to the injury. There are certain caveats here, however. It is hard to dissect process important for reprogramming from other processes such as the innate immune response, cell migration, and other consequences of injury. It is also unknown to what extent these processes are conserved in mammals. Still, dedifferentiation of cells to form proliferating progenitor cells is interesting, and systems such as skeletal muscle, limb and tail regeneration or dorsal iris epithelium during lens regeneration should be studied further with screens designed to find the key players involved.

The main challenge facing elucidation of nuclear reprogramming mechanisms using the conventional approaches, and potential solutions

The main problem with current studies investigating nuclear reprogramming mechanisms is the lack of material due to the low frequencies of reprogramming using artificial methods. Conventional approaches entail isolating and expanding reprogrammed cells in strongly selective culture conditions [e.g. in cell fusion experiments (Tada *et al.*, 1997; Cowan *et al.*, 2005) hybrid clones were isolated by antibiotic resistance and expanded]. Analysing such material, however, does not allow discrimination between the epigenetic changes necessary for the induction of reprogramming versus those that happen independently of such induction; i.e. it does not allow the study of reprogramming as it is happening.

How can the study of this process be facilitated? One strategy is to use easily reprogrammable cells, such as cells differentiated from ES cells in culture (Blelloch *et al.*, 2006; Silva *et al.*, 2006). Perhaps the initial focus should be on cultured cells instead of later primary cells, as these will still have strong epigenetic regulation, and thus would be harder to reprogram. Experiments with cultured cells should yield more reprogrammed material.

Additionally, it would be possible to use chromatin modifying drugs such as trichostatin A and 5-aza-2'-deoxycytidine to make the chromatin less condensed and more accessible. Factors required for activating the *Oct-3/4* gene are unknown, but recently it has been shown that two chromatin modifying drugs can activate the *Oct-3/4* gene in cells (Hattori *et al.*, 2004). These two drugs, trichostatin A (TSA) and 5-aza-2'-deoxycytidine

(5-aza-dC), which inhibit histone deacetylation and DNA methylation respectively, are thought to make the chromatin structure more open and consequently the *Oct-3/4* gene easier to activate. However, such drug treatment is quite toxic to the cells as well as being non-specific (these drugs reactivate many genes including those not associated with an ES cell phenotype (S Sullivan, unpublished data). Tsuji-Takayama *et al.* (2004) have recently shown that treatment of differentiated ES cells with a similar chemical to 5-aza-dC, called 5-azacytidine, causes the up-regulation of stem cell marker genes *Oct-3/4*, *Nanog* and *Sox2*. As with Hattori's work, the expression of genes associated with differentiated cells were not studied, and it is expected that these too will be up-regulated. It will be very interesting to screen for more specific drugs that increase the frequency of reprogramming.

Thirdly, although the reason is unknown, cell cycle synchronization by serum starvation makes murine embryonic fibroblasts (MEF) more easily reprogrammed both by NT (Campbell, 1996) or cell fusion (Sullivan *et al.*, 2006). This strategy could also facilitate reprogramming studies.

Can one learn about reprogramming and improve its efficiency by transposing conditions between the three reprogramming methods?

In order to learn from experiments using the three different methods to deduce the reprogramming mechanism(s) and improve their efficiencies, it is necessary to compare and contrast observations from them. At present, it is difficult to dissect the important events such as changes in gene regulation and chromatin structure during the reprogramming processes due to the inefficiency of all three methods, but some hints can be gathered from existing kinetic, gene expression, and cell cycle data. The kinetics of reprogramming appears to be very similar between NT and cell fusion. Somatic cell-derived transgenic Oct-3/4 is expressed within 24 h after NT and cell fusion (Sullivan and Egli, unpublished data). In contrast, reprogramming experiments using viral transduction have shown that stem cell genes *Alkaline Phosphatase*, *SSEA-1*, and *Nanog* are not highly expressed until 2–3 weeks post-infection (Blelloch *et al.*, 2007; Maherali *et al.* 2007; Meissner *et al.* 2007; Okita *et al.* 2007; Wernig *et al.* 2007), indicating that reprogramming proceeds at a slower pace with this method. The need to synthesize the four reprogramming genes *de novo* can only partially explain the slower kinetics of reprogramming using the viral transduction method. It is likely that other proteins that facilitate the induction of reprogramming during NT and cell fusion are missing, or that the entire transcriptional programme required for reprogramming, which is more completely expressed by the oocyte during NT or the ES cell during cell fusion, is vast and requires a substantial amount of time to execute. For example, demethylation of promoters of endogenous genes such as Oct-3/4 may occur very slowly during reprogramming by viral transduction if factors required for active demethylation are not produced as they are thought to be during NT (Yamazaki *et al.* 2006).

The two pluripotency genes used in the iPS cell viral transduction approach, *Oct-3/4* and *Sox2*, are expressed in oocytes (Avilion *et al.*, 2003; Monti *et al.*, 2006) and mouse ES cells (Yamanaka, 2007), suggesting that their roles in establishing and/or maintaining pluripotency are conserved in all three reprogramming approaches. Yamanaka posits that c-Myc may make the chromatin more accessible to transcription factors by binding to many sites in the genome and inducing histone deacetylation in addition to promoting self-renewal, as it does in murine ES cells (Cartwright *et al.* 2005; Yamanaka, 2007). c-Myc is expressed in oocytes (Naz *et al.* 1994) but is not highly expressed in mouse ES cells (Blelloch *et al.*, 2007). However, a functionally equivalent family member, n-Myc, is expressed and can substitute for c-Myc in iPS cell transduction (Blelloch *et al.*, 2007). Thus, Myc proteins may stimulate self-renewal in iPS cell transduction, cell fusion and NT. *Klf-4* is highly expressed in mouse ES cells (Yamanaka, 2007) and thus may play a role in reprogramming during cell fusion.

Cell cycle synchronization of the somatic cells into G_0/G_1 or G_2/M prior to NT or cell fusion increases the efficiency of reprogramming (Campbell *et al.*, 1996; Sullivan *et al.*, 2006). This effect is attributable to avoiding the aneuploidy or chromosomal damage risked by nuclear transfer or cell fusion during S phase. Yamanaka used unsynchronized cells in the iPS cell transduction experiments because active cell division is a requirement for infection by Moloney retrovirus. Egli and coworkers determined that a zygote arrested in mitosis can reprogram a somatic nucleus while an interphase zygote cannot (Egli *et al.*, 2007). A major difference between a mitotic zygote and an interphase zygote is that the nuclear membrane has broken down in the mitotic zygote. Therefore, it is possible that factors required for reprogramming are sequestered in the nucleus during interphase and released during mitosis. In cell fusion in mice, ES cells in G_2/M phase were the most effective at reprogramming, suggesting that key reprogramming activities at that stage of the cell cycle (Sullivan *et al.*, 2006).

Now there is the opportunity to use observations made in one method of reprogramming to try to improve the other methods. For example, will overexpressing some or all of the four Yamanaka factors in ES cells make reprogramming by cell fusion more efficient? The best evidence that this might be the case is given by Silva and coworkers. They reported elevated frequencies of reprogramming in a cell fusion system where *Nanog*, a pluripotency gene not necessary for iPS cell formation by viral transduction, was overexpressed in the ES cell fusion partner (Silva *et al.*, 2006). High Nanog levels may assist the induction of reprogramming indirectly as positive feedback circuits involving Nanog elevate Oct-3/4 and Sox2 levels (Loh *et al.*, 2006).

It will also be interesting to introduce c-*Myc* and *Klf-4* transgenically into cells to be reprogrammed by NT or cell fusion, to see if this increases the frequency of reprogramming; however, as these gene are both oncogenes, the resultant cells should be tested for epigenetic and genetic abnormalities. There is an additional caveat with this approach: what is learned from reprogramming genetically manipulated, cultured cells may not immediately inform the process of reprogramming normal primary somatic cells, which still have all epigenetic regulatory processes intact. It is, however, a first step towards reprogramming primary cells and should give enough material to untangle the various mechanisms.

Slow demethylation or chromatin re-structuring may be why Yamanaka's viral transduction method proceeds more slowly than NT or cell fusion. This seems likely, given that the other two methods have other factors that could potentially speed up these processes. For example, Yamazaki and coworkers found that even in NT, demethylation of the *Oct-4* promoter proceeds gradually and is probably a result of both active and passive mechanisms for demethylation (Yamazaki *et al.*, 2006). Yamanaka's four factors may not be sufficient to induce active demethylation, and may be dependent on the passive mechanism alone, causing slower reprogramming. Overexpression of de-novo methyl-transferase genes such as *Dnmt-1* or *Dnmt-3* might facilitate the process. Alternatively, if chromatin remodelling is the rate-limiting step, small molecule HDAC inhibitors could expedite reprogramming.

In the future, determining the list of genes that are up-regulated in ES cells during G_2/M phase or proteins that are localized in the nucleus during interphase in zygotes will significantly concentrate the search for genes necessary for reprogramming. Additionally, Yamanaka's work suggests that transcription factor libraries may be the most fruitful source of reprogramming factors.

Currently, it seems reasonable that all three reprogramming methods share a general mechanism involving chromatin remodelling to allow changes in gene expression as the first step, followed by changes to prevent cell death. The last step would be the induction of pluripotency. It also seems likely that the genes used to induce pluripotency are the same in all three methods, while there could be different molecular pathways to cell immortalization and altering DNA accessibility.

Conclusion

NT is the only reprogramming technique known not to require addition of foreign genes to induce restoration of developmental potential. Furthermore, it is still the only method can restore pluripotency without a high risk of oncogenesis. Thus, NT remains a very important system for studying reprogramming. Efficiency by this and the other two methods discussed is, however, still very low and the lack of material limits efforts to identify important factors for reprogramming induction. All three methods (NT, cell fusion, and iPS cell transduction) should be perused so that conditions optimal in one system can be implemented in the others to try to improve reprogramming frequencies. The four iPS cell factors can be introduced into cells that are to be used in NT and cell fusion experiments with the hope of increasing the frequency of reprogramming. It is hoped this will provide more material to study mechanisms and so help understanding of reprogramming. The scarcity of tissues and organs for transplantation, as well as the need for pluripotent stem cells to develop in-vitro models of human disease and development, compel further study of reprogramming mechanisms.

Acknowledgements

SS is funded by a fellowship from the Harvard Stem Cell Research Foundation. JI is funded by a fellowship from the New York Stem Cell Foundation. AU and HA are funded by Health and Labour Sciences Research Grants, Japan. The authors thank Esther Son, Kit Rodolfa, Katelyn Foley, and Gabriella Boulting for proofreading the manuscript.

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Received 27 July 2007; refereed 12 September 2007; accepted 17 October 2007.