GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Short Communication

Forced expression of *OCT4* influences the expression of pluripotent genes in human mesenchymal stem cells and fibroblasts

C.S. Palma[1], M.A. Tannous[1], T.M. Malta[1], E.M.S. Russo[1,2], D.T. Covas[1,3] and V. Picanço-Castro[1]

[1]Instituto Nacional de Ciência e Tecnologia em Células-Tronco e Terapia Celular, Hemocentro de Ribeirão Preto, Ribeirão Preto, SP, Brasil
[2]Departamento de Análises Clínicas Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil
[3]Departamento de Clínica Médica, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil

Corresponding author: V. Picanço-Castro
E-mail: virginia.picanco.castro@gmail.com

Genet. Mol. Res. 12 (2): 1054-1060 (2013)
Received September 13, 2012
Accepted February 29, 2013
Published April 2, 2013
DOI http://dx.doi.org/10.4238/2013.April.2.22

ABSTRACT. Genetic reprogramming of adult cells to generate induced pluripotent stem (iPS) cells is a new and important step in sidestepping some of the ethical issues and risks involved in the use of embryonic stem cells. iPS cells can be generated by introduction of transcription factors, such as OCT4, SOX2, KLF4, and CMYC. iPS cells resemble embryonic stem cells in their properties and differentiation potential. The mechanisms that lead to induced pluripotency and the effect of each transcription factor are not completely understood. We performed a critical evaluation of the effect of overexpressing *OCT4* in mesenchymal stem cells and fibroblasts and found that *OCT4* can activate the expression of other stemness genes, such as *SOX2*, *NANOG*, *CMYC*, *FOXD3*, *KLF4*, and *βCATENIN*, which are not normally or are very weakly expressed in mesenchymal stem cells. Transient expression of *OCT4* was also performed to evaluate

whether these genes are affected by its overexpression in the first 48 h. Transfected fibroblast cells expressed around 275-fold more *OCT4* than non-transfected cells. In transient expression, in which cells were analyzed after 48 h, we detected only the up-regulation of *FOXD3*, *SOX2*, and *KLF4* genes, suggesting that these genes are the earlier targets of *OCT4* in this cellular type. We conclude that forced expression of *OCT4* can alter cell status and activate the pluripotent network. Knowledge gained through study of these systems may help us to understand the kinetics and mechanism of cell reprogramming.

Key words: Embryonic stem cell; Pluripotency; OCT4 targets

INTRODUCTION

Embryonic stem cells (ESC), derived from the inner cell mass of mammalian blastocysts, are termed pluripotent cells due to their ability to self-renew and to differentiate into any cell type within the organism (Pan and Thomson, 2007; Lengner et al., 2008). The development of ESC-like cells, induced pluripotent stem (iPS) cells, from somatic cells is an important step in overcoming some of the ethical issues involving the use of ESC. iPS cells can be generated by the addition of pluripotent factors such as OCT4, SOX2, KLF4, and CMYC and resemble ESC in their properties and differentiation potential (Takahashi et al., 2007). However, the mechanisms that lead to the reprogramming of somatic cells are not completely understood and the specific contribution of each reprogramming factor remains unknown.

Recent data suggest that the pluripotent properties may be regulated by a small number of ESC-specific transcription factors such as OCT4, SOX2 and NANOG, whose expression is downregulated during embryogenesis (Boyer et al., 2005).

The transcription factor OCT4, also known as OCT3, OCT3/4 and POU5F1, is a member of the POU (Pit-Oct-Unc) family of transcription factors and activates the expression of its target genes by binding to a specific sequence on the promoter region - ATGCAAAT/TACGTTTA. OCT4 is highly expressed during oogenesis, in the inner cell mass of the blastocyst (ESC) and in the epiblast (Zago, 2006), and becomes less expressed as cell differentiation proceeds. Apparently, OCT4 functions in coordination with NANOG and SOX2 to maintain ESC pluripotency (Chambers, 2004).

On the basis of the hypothesis that one pluripotent factor can activate other transcription factors, we delivered the *OCT4* gene into human fibroblasts and mesenchymal stem cells (MSC) to evaluate the effects of *OCT4* overexpression. We found that the transient high levels of *OCT4* activated three pluripotent genes (*KLF4*, *SOX2* and *FOXD3*), whereas stable high levels of *OCT4* expression activated *KLF4*, *SOX2*, *FOXD3*, and also *NANOG* and *CMYC*. These results provide insights into the understanding of the mechanisms of iPS generation and may help to improve the stemness of MSC/fibroblasts by genetic modification with stemness-related genes.

MATERIAL AND METHODS

Cell culture

Human MSC, obtained from bone marrow donors of the Regional Blood Center of Ri-

beirão Preto (CEP HCRP No. 11429/2008), and the human fibroblast cell line CCD-27Sk (ATCC CRL-1475) were grown to a concentration of 5 to 6 x 10^5 cells/mL in 75-cm² flasks and maintained in Dulbeccos's modified Eagle's medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Seromed, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma, USA).

Plasmid construction

The OCT4 transcription factor was amplified by *Pfu* polymerase chain reaction (PCR) of cDNA from human embryonic stem cells. The open reading frame of human *OCT4* was cloned into pLenti-cPPT-CIGWS, which is a bicistronic vector coding for EGFP. The cloned fragment was verified by sequencing.

Lentiviral production

The plasmid DNA carrying the *OCT4* gene was introduced into Hek-293T (ATCC CRL-11268) cells by a triple cotransfection with the packaging construct pCMVΔR8.91, and the pseudotyping construct pMD2.VSVG, which codes for vesicular stomatitis virus glycoprotein G. Transfection of monolayers with the DNA mixture was done using Lipofectamine™ 2000 (Invitrogen, USA). Six hours after transfection, the supernatants were replaced by fresh medium and cells were incubated for an additional 48 h. The culture supernatants containing pseudovirions were collected and stored at -80°C. Virus titers were determined in 293T cells by EGFP percentage using fluorescence-activated cell sorting (FACS) on a FACSCalibur flow cytometer (BD Biosciences, USA). The lentiviral titers were expressed as 293T-transducing units per milliliter.

Lentiviral infection

MSC were seeded at 1 x 10^5 cells on a 6-well plate. The viruses were added to the mesenchymal cells at a multiplicity of infection of 10, and the plate incubated overnight. Twelve hours after transduction, the virus-containing medium was replaced by DMEM supplemented with 10% FBS.

Transfection

CCD-27Sk fibroblasts were seeded at 5 x 10^5 cells per 100-mm dish 1 day before the transfection. Ten micrograms of the pLenti-cPPT-CIGWS was introduced into monolayers by transfection using Lipofectamine™ 2000 following the manufacturer protocol.

Flow cytometry

Mesenchymal cells and fibroblasts transduced/transfected with *OCT4* lentiviral vector were trypsinized, and FACS analysis was performed based on forward and side scatters. GFP fluorescence was measured using a FACSCalibur flow cytometer. All cell populations were sorted twice to optimize cell purity. Percentages of GFP-positive cells were determined by analyzing at least 50,000 cells per sample.

Real-time PCR assays

Total RNA of the transduced and non-transduced cells was purified with the RNeasy® Micro-kit (Qiagen, USA). One microgram of total RNA was used for the reverse transcription reaction using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA), according to manufacturer instructions. Quantitative PCR was performed with TaqMan (Invitrogen) and analyzed with the 7500 Real-time PCR system (Applied Biosystems). Gene expression was normalized to the endogenous gene *GAPDH*.

RESULTS

Forced expression of *OCT4* in adult cells was done using a third-generation lentiviral vector (1054-CMV-OCT4) constructed in our laboratory. We analyzed both stable and transient expression. For stable *OCT4* expression, cells were incubated overnight with the 1054-CMV-OCT4 virus, and for transient *OCT4* expression, cells were transfected with 10 µg 1054-CMV-OCT4 plasmid DNA. The cell lines used in this study were the fibroblast cell line CCD-27Sk and MSC obtained from bone marrow donors. The expression of the *GFP* gene was used to determine the efficiency of *OCT4* delivery. The RNA from transduced and transfected cells was extracted and the cDNA was submitted to real-time PCR. Expression was compared to ESC.

Transduced cells showed no changes in morphology (Figure 1). The cells expressed around 225-fold more *OCT4* compared to the non-transduced cells (N = 3). We also found that *OCT4*-expressing cells expressed other stemness genes, namely *SOX2*, *NANOG*, *CMYC*, *FOXD3*, and *KLF4*, which are not normally expressed or expressed in very low amounts in MSC. The expression of *βCATENIN* was slightly increased with overexpression of *OCT4* (Figure 2I).

In transient expression, the *OCT4*-transfected cells expressed around 275-fold more *OCT4* compared to the non-transfected cells. However, in transient expression in which cells were analyzed after 48 h, increased expression was also observed for the *SOX2*, *KLF4*, and *FOXD3* genes, respectively, 31-, 82-, and 8.7-fold compared to control cells, suggesting that these genes may be initial targets of *OCT4*. There was no significant increase in the expression of the other genes (Figure 2II).

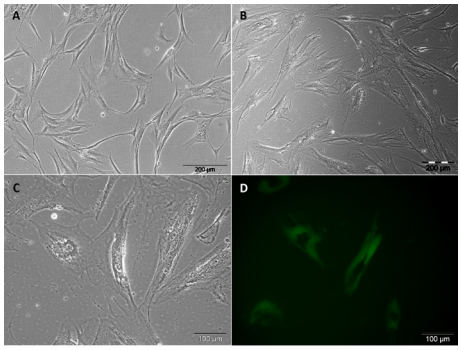

Figure 1. Morphology of control mesenchymal stem cells (MSCs) and transduced MSCs with the lentiviral vector 1054-CMV-OCT4. Images from optical microscopy. **A.** Non-transduced MSC (control), phase contrast microscopy, 10X magnification. **B.** Transduced MSC, phase contrast microscopy, 10X magnification. **C.** Transduced MSC, phase contrast microscopy, 20X magnification. **D.** Transduced MSC, EGFP expression, fluorescence microscopy, 20X magnification.

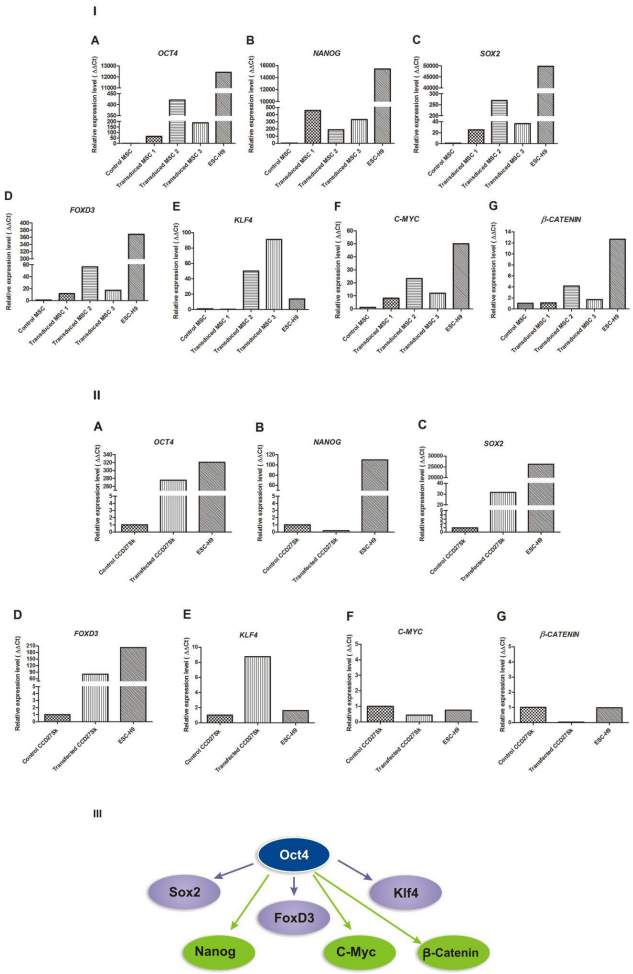

Figure 2. I. Gene expression analysis of transduced mesenchymal stem cells (MSCs) with the lentiviral vector 1054-CMV-OCT4 (transduced MSC) and ES-H9, in comparison to non-transduced cells (control MSC). Transduced cells 1, 2 and 3 exhibited, respectively, 6, 11 and 27% EGFP expression, evaluated by flow cytometry. Quantification of relative expression level of the genes OCT4, CMYC, SOX2, FOXD3, KLF4, β-CATENIN, and NANOG through real-time PCR and ΔΔCt methodology. **II.** Gene expression analysis of transfected CCD-27Sk with the lentiviral vector 1054-CMV-OCT4 (CCD27Sk-OCT4) in comparison to non-transfected cells (control CCD27Sk). Transfected cells exhibited 25% EGFP expression, evaluated by flow cytometry. Quantification of relative expression level of the genes OCT4, CMYC, SOX2, FOXD3, KLF4, β-CATENIN, and NANOG through real-time PCR and ΔΔCt methodology. **III.** Schematic representation of OCT4 targets.

DISCUSSION

The pluripotent cell state is regulated by the action of a gene-regulatory network centered around OCT4, NANOG, and SOX2. These three transcription factors bind at closely localized sites in ESC chromatin, and they have positive/negative feedback interactions that stabilize expression of the three genes.

The *OCT4* gene plays a critical role in the maintenance of pluripotency and its cellular expression levels are precisely regulated, to ensure continuity in the germline or differentiation of various cell types (Pan et al., 2002). However, the specific role of *OCT4* is not well known. To better understand transcription regulation by this factor, we overexpressed *OCT4* in fibroblast-like cells.

Our results indicated that the *OCT4* gene was not expressed in human MSC or in fibroblasts, contradicting data from previous reports (Pochampally et al., 2004; Izadpanah et al., 2006; Greco et al., 2007; Go et al., 2008; Riekstina et al., 2009) but in agreement with other studies (Berg and Goodell, 2007; Lengner et al., 2007; Liedtke et al., 2007; Kaltz et al., 2008). This discrepancy found in the literature is due to the fact that *OCT4* has at least 4 protein isoforms (OCT4A, OCT4B-190, OCT4B-265, and OCT4B-164) generated by an alternative splicing and alternative translation initiation (Wang and Dai, 2010). OCT4A is a transcription factor responsible for the pluripotency properties of ESC, which is the gene isoform evaluated in our study. We found that *OCT4* ectopic expression is able to activate the endogenous genes *SOX2*, *KLF4*, *FOXD3*, *NANOG*, and *CMYC*, which are not naturally expressed in mesenchymal cells or fibroblasts.

Our results also showed that the expression levels of *SOX2*, *KLF4* and *FOXD3* target genes tended to increase after 48 h of transfection, and 120 h after transduction, we could detect the same three transcription factors plus *NANOG* and *CMYC* (Figure 2III). We speculate that *SOX2*, *KLF4* and *FOXD3* are initial target genes of OCT4. All these transcription factors contribute to the maintenance of pluripotency. *FOXD3* codes for a transcription factor that represses essential targets for the maintenance of undifferentiated ESC lines. SOX2 binds to DNA cooperatively with OCT4 at non-palindromic sequences to activate the transcription of key pluripotency factors (Chambers and Tomlinson, 2009). KLF4 is essential for somatic cell reprogramming. In addition, KLF4 seems to play a redundant role along with other Klf family proteins in ESC self-renewal. CMYC induces DNA replication and cell cycle progression, and modulates the ESC epigenome, promoting the opening of chromatin architecture to the action of pluripotent factors (Scheper and Copray, 2009; Varlakhanova et al., 2010). NANOG expression is restricted to pluripotent cells and is downregulated upon differentiation, but little is known about the regulation of NANOG expression. OCT4 and SOX2 are the major transcription factors that bind to the NANOG promoter *in vitro* and *in vivo* to promote NANOG transcription (Boyer et al., 2005). Another pluripotent factor that can also activate NANOG expression is FOXD3, and that can explain the later activation of *NANOG* by the ectopic *OCT4* gene. *OCT4* and *FOXD3* may have contributed to *NANOG* activation in our cells. Our results demonstrate that *OCT4* can interact with and activate other pluripotent genes, contributing to a better understanding of the mechanisms involved in reprogramming of somatic cells, so that in the future, iPS cells can be generated with greater efficiency and safety.

ACKNOWLEDGMENTS

Research supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). We thank Sandra Navarro Bresciani for her help with the figures.

REFERENCES

Berg JS and Goodell MA (2007). An argument against a role for Oct4 in somatic stem cells. *Cell Stem Cell* 1: 359-360.

Boyer LA, Lee TI, Cole MF, Johnstone SE, et al. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* 122: 947-956.

Chambers I (2004). The molecular basis of pluripotency in mouse embryonic stem cells. *Cloning Stem Cells* 6: 386-391.

Chambers I and Tomlinson SR (2009). The transcriptional foundation of pluripotency. *Development* 136: 2311-2322.

Go MJ, Takenaka C and Ohgushi H (2008). Forced expression of Sox2 or Nanog in human bone marrow derived mesenchymal stem cells maintains their expansion and differentiation capabilities. *Exp. Cell Res.* 314: 1147-1154.

Greco SJ, Liu K and Rameshwar P (2007). Functional similarities among genes regulated by OCT4 in human mesenchymal and embryonic stem cells. *Stem Cells* 25: 3143-3154.

Izadpanah R, Trygg C, Patel B, Kriedt C, et al. (2006). Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue. *J. Cell Biochem.* 99: 1285-1297.

Kaltz N, Funari A, Hippauf S, Delorme B, et al. (2008). *In vivo* osteoprogenitor potency of human stromal cells from different tissues does not correlate with expression of POU5F1 or its pseudogenes. *Stem Cells* 26: 2419-2424.

Lengner CJ, Camargo FD, Hochedlinger K, Welstead GG, et al. (2007). Oct4 expression is not required for mouse somatic stem cell self-renewal. *Cell Stem Cell* 1: 403-415.

Lengner CJ, Welstead GG and Jaenisch R (2008). The pluripotency regulator Oct4: a role in somatic stem cells? *Cell Cycle* 7: 725-728.

Liedtke S, Enczmann J, Waclawczyk S, Wernet P, et al. (2007). Oct4 and its pseudogenes confuse stem cell research. *Cell Stem Cell* 1: 364-366.

Pan G and Thomson JA (2007). Nanog and transcriptional networks in embryonic stem cell pluripotency. *Cell Res.* 17: 42-49.

Pan GJ, Chang ZY, Schöler HR and Pei D (2002). Stem cell pluripotency and transcription factor Oct4. *Cell Res.* 12: 321-329.

Pochampally RR, Smith JR, Ylostalo J and Prockop DJ (2004). Serum deprivation of human marrow stromal cells (hMSCs) selects for a subpopulation of early progenitor cells with enhanced expression of OCT-4 and other embryonic genes. *Blood* 103: 1647-1652.

Riekstina U, Cakstina I, Parfejevs V, Hoogduijn M, et al. (2009). Embryonic stem cell marker expression pattern in human mesenchymal stem cells derived from bone marrow, adipose tissue, heart and dermis. *Stem Cell Rev.* 5: 378-386.

Scheper W and Copray S (2009). The molecular mechanism of induced pluripotency: a two-stage switch. *Stem Cell Rev.* 5: 204-223.

Takahashi K, Tanabe K, Ohnuki M, Narita M, et al. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131: 861-872.

Varlakhanova NV, Cotterman RF, deVries WN, Morgan J, et al. (2010). myc maintains embryonic stem cell pluripotency and self-renewal. *Differentiation* 80: 9-19.

Wang X and Dai J (2010). Concise review: isoforms of OCT4 contribute to the confusing diversity in stem cell biology. *Stem Cells* 28: 885-893.

Zago MA (2006). Células-Tronco: Origens e Propriedades. In: Células-Tronco: A Nova Fronteira da Medicina (Covas DT and Zago MA, eds.). Editora Atheneu, São Paulo, 3-20.