Stem Cell Research (2014) 12, 730–741

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Mechanistic elements and critical factors of cellular reprogramming revealed by stepwise global gene expression analyses

Sung-Jin Park[a,1], Hock Chuan Yeo[b,1], Nam-Young Kang[a,1], Hanjo Kim[c,2], Joyce Lin[b], Hyung-Ho Ha[c,3], Marc Vendrell[a,4], Jun-Seok Lee[c,5], Yogeswari Chandran[a], Dong-Yup Lee[b,d,*], Seong-Wook Yun[a,**], Young-Tae Chang[a,c,***]

[a] *Laboratory of Bioimaging Probe Development, Singapore Bioimaging Consortium, Agency for Science, Technology and Research, Singapore 138667, Republic of Singapore*
[b] *Bioinformatics Group, Bioprocessing Technology Institute, Agency for Science, Technology and Research, Singapore 138668, Republic of Singapore*
[c] *Department of Chemistry & NUS MedChem Program of Life Sciences Institute, National University of Singapore, Singapore 117543, Republic of Singapore*
[d] *Department of Chemical and Biomolecular Engineering, National University of Singapore, Singapore 117576, Republic of Singapore*

Received 31 May 2013; received in revised form 21 February 2014; accepted 17 March 2014
Available online 25 March 2014

Abstract A better understanding of the cellular and molecular mechanisms involved in the reprogramming of somatic cells is essential for further improvement of induced pluripotent stem (iPS) cell technology. In this study, we enriched for cells actively undergoing reprogramming at different time points by sorting the cells stained with a stem cell-selective fluorescent chemical

Abbreviations: CDy1, compound of designation yellow 1; DEG, differentially expressed gene; FDR, false discovery rate; OSKM, Oct4, Sox2, Klf4, c-Myc; PDGF, platelet derived growth factor.

* Correspondence to: D.-Y. Lee, Department of Chemical and Biomolecular Engineering, National University of Singapore, Singapore 117576, Republic of Singapore. Fax: +65 6779 1936.
** Correspondence to: S.-W. Yun, Laboratory of Bioimaging Probe Development, Singapore Bioimaging Consortium, Agency for Science, Technology and Research, Singapore 138667, Republic of Singapore. Fax: +65 6478 9957.
*** Correspondence to: Y.-T. Chang, Department of Chemistry & NUS MedChem Program of Life Sciences Institute, National University of Singapore, Singapore 117543, Republic of Singapore. Fax: +65 6516 1691.
E-mail addresses: cheld@nus.edu.sg (D.-Y. Lee), yun_seong_wook@sbic.a-star.edu.sg (S.-W. Yun), chmcyt@nus.edu.sg (Y.-T. Chang).
URL: http://ytchang.science.nus.edu.sg (Y.-T. Chang).
[1] These authors contributed equally to this work.
[2] Present address: R&D Centre, EQUISnZAROO Co., Ltd., Seongnam 463-400, Republic of Korea.
[3] Present address: College of Pharmacy, Sunchon National University, Sunchon 540-742, Republic of Korea.
[4] Present address: MRC Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh, EH16 4TJ, UK.
[5] Present address: Future Convergence Research Division, Biomolecules Function Research Centre, Korea Institute of Science and Technology, Seoul 136-791, Republic of Korea.

http://dx.doi.org/10.1016/j.scr.2014.03.002

probe CDy1 for their global gene expression analysis. Day-to-day comparison of differentially expressed genes showed highly dynamic and transient gene expressions during reprogramming, which were largely distinct from those of fully-reprogrammed cells. An unbiased analysis of functional regulation indicated robust modulation of concurrent programs at critical junctures. Globally, transcriptional programs involved in cell proliferation, morphology and signal transduction were instantly triggered as early as 3 days-post-infection to prepare the cell for reprogramming but became somewhat muted in the final iPS cells. On the other hand, the highly coordinated metabolic reprogramming process was more gradually activated. Subsequent network analysis of differentially expressed genes indicated PDGF-BB as a core player in reprogramming which was verified by our gain- and loss-of-function experiments. As such, our study has revealed previously-unknown insights into the mechanisms of cellular reprogramming.

Introduction

Since the first report of induced pluripotent stem (iPS) cells generated by the expression of four transcription factors Oct4, Sox2, Klf4 and c-Myc (OSKM) in fibroblasts (Takahashi and Yamanaka, 2006), there has been a variety of technical improvements to generate iPS cells more efficiently. Different combinations of reprogramming transcription factors or addition of synthetic chemicals that influence epigenetic regulation and signaling pathways have been discovered to enhance the reprogramming efficiency and quality of stem cells (Feng et al., 2009b; Han et al., 2010; Lin et al., 2009). However, the mechanism of reprogramming, especially at the early phases, remains elusive. One of the reasons for the scant knowledge about reprogramming mechanisms is the very low efficiency of reprogramming. Only a small number of cells, which is undergoing reprogramming, need to be isolated out of heterogeneous cell populations for the analysis. Although the expression of fluorescent proteins using Oct4 or Nanog promoters has been used to detect and isolate mature iPS cells in 2–4 weeks of time, there has been no efficient way to probe the cells undergoing reprogramming at early phases. Profiling gene expression patterns during reprogramming has previously been attempted using unsorted mixed populations of secondary mouse embryonic fibroblasts (MEFs) harboring doxycycline-inducible OSKM genes (Samavarchi-Tehrani et al., 2010) or partially reprogrammed cell lines which were not actively undergoing reprogramming (Mikkelsen et al., 2008). Recently, Buganim et al. identified single cells which turn into iPS cells and analyze expression profiles of 48 genes in those cells (Buganim et al., 2012), while Polo et al. analyzed global gene expression profiles of the cells enriched based on SSEA-1 and Thy1 expressions (Polo et al., 2012) and Hansson et al. analyzed proteome changes during reprogramming under dox-inducible transgenic system (Hansson et al., 2012). The reprogramming mechanisms newly revealed by these studies demonstrate the necessity of more tools with which the rare cells undergoing reprogramming can be isolated.

We previously reported a fluorescent chemical probe CDy1, which selectively stains living embryonic stem (ES) cells of both human and mouse origin and enables the isolation of ES cells from a mixture with fibroblasts by fluorescence-activated cell sorting (FACS) (Im et al., 2010; Kang et al., 2011). CDy1 detects iPS cells as well, at both early and late phases of reprogramming as demonstrated using the cells of a transgenic mouse that express GFP under the control of Oct4 promoter. When the mouse fibroblasts were transfected with retroviral vectors encoding OSKM and then incubated with CDy1, some colonies were brightly stained at 10 days post infection (dpi) before GFP expression, which eventually turned into GFP-expressing colonies at a later time. In another study, we demonstrated that CDy1 can be used as an iPS cell reporter even at 7 dpi in a high throughput screening of chemicals designed for the development of reprogramming enhancer (Vendrell et al., 2012). These findings led us to hypothesize that cells undergoing reprogramming could be isolated at different time points during reprogramming using CDy1 and their stepwise global gene expression analysis would enable the identification of key mechanisms, pathways or molecules that play important roles in cellular reprogramming. Based on this hypothesis, we identified differentially expressed genes (DEGs) in the CDy1-positive cells harvested at several early time points of reprogramming starting from as early as 3 dpi and analyzed the data using various bioinformatics tools. A functional transcriptomic analysis of the data revealed an unprecedented sequence of cellular mechanisms of reprogramming and a DEG network analysis identified PDGF-BB as a critical factor for the process.

Materials and methods

Cell culture

MEFs were cultured on gelatin-coated dish in high-glucose Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM non-essential amino acids and 0.1% β-mercaptoethanol. To be used as feeder, the cells were treated with mitomycin C (10 μg/ml) for 2 h and washed with PBS. iPS cells were cultured on these feeder cells in DMEM containing 20% knock-out serum replacement (KOSR, Invitrogen), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% non-essential amino acids and 100 U/ml leukemia inhibitory factor (LIF, Chemicon).

iPS cell generation

MEFs prepared from B6;CBATg(Pou5f1-EGFP)2Mnn/J mouse (Jackson Laboratory) were infected by pMX-Oct4, Sox2, Klf4 and c-Myc expressing retrovirus with 10 μg/ml polybrene (Sigma) in normal MEF culture medium for 1 day and the medium was replaced with iPS cell culture medium the next day (1 dpi). At 2 dpi, the infected cells were seeded on