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Cyclin D1 acts as a barrier to pluripotent reprogramming by promoting neural progenitor fate commitment

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ABSTRACT

A short G1 phase is a characteristic feature of the cell cycle structure of pluripotent cells, and is reestablished during Yamanaka factor-mediated pluripotent reprogramming. How cell cycle control is adjusted to meet the requirements of pluripotent cell fate commitment during reprogramming is less well understood. Elevated levels of cyclin D1 were initially found to impair pluripotency maintenance. The current work further identified Cyclin D1 to be capable of transcriptionally upregulating *Pax6*, which promoted reprogramming cells to commit to a neural progenitor fate rather than a pluripotent cell fate. These findings explain the importance of reestablishment of G1-phase restriction in pluripotent reprogramming.

1. Introduction

Pluripotent cells in the inner cell mass exhibit a shortened G1 phase and lack G1 checkpoint regulation [1,2], while neural stem/progenitor cells (NPCs) have a tightly regulated and lengthened G1 phase associated with their self-renewal and differentiation status [3–5]. For example, Lange et al., have recently shown that G1 lengthening is critically regulated during the expansion and differentiation of NPCs into neurons, and Artegiani et al., demonstrated that overexpression of cell cycle regulators cdk4 or cyclin D1 can trigger the expansion of NPCs in adult mouse brain [4,6]. Others have shown that cyclin A2 critically regulates the cell cycle of pluripotent embryonic stem (ES) cells [2,7–9], and a shortened G1 phase protects ES cells from external signals that can induce endodermal or neuroectodermal differentiation [10]. These findings suggest that cell cycle state is modulated during the differentiation process in order to meet the requirements of cell fate commitment during development. However, the key regulators that link cell cycle remodeling and cell fate commitment remain to be explored.

Pluripotent reprogramming, achieved by introducing Yamanaka factors (Oct4, Sox2, c-Myc and Klf4) into somatic cells, has been used to study the pathogenesis of inherited genetic diseases and to identify novel drug targets [11–13]. Recent studies have further demonstrated that Yamanaka factor-mediated fibroblast reprogramming can generate not only induced pluripotent stem (iPS) cells, but also cells that possess features of multipotent hematopoietic progenitors [14] or NPCs [15,16]. Previous work by Ruiz et al., has already shown that cell cycle features of ES cells are acquired during pluripotent reprogramming induced by Yamanaka factors [17]. These findings imply that the fate of cells undergoing reprogramming may be tightly associated with cell cycle remodeling. The present study investigated whether regulators involved in reestablishment of cell cycle structures of ES cells or NPCs during Yamanaka factor-mediated reprogramming are also cell fate

Abbreviations: ES cells, embryonic stem cells; iPS cells, induced pluripotent stem cells; NPCs, neural stem/progenitor cells; EGFP, enhanced green fluorescent protein; SSEA, stage specific embryonic antigen

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determinants. We found that sustained production of cyclin D1, a G1 phase regulator, during reprogramming possibly acts as a barrier to the successful generation of iPS cells by promoting neural progenitor fate commitment through transcriptionally upregulating *Pax6*.

2. Materials and methods

2.1. Generation of Oct4-EGFP-positive iPS cells

Oct4-EGFP-positive iPS cells were generated as previously described [11,12]. Briefly, for retrovirus packaging and production, pMXs retroviral vectors encoding Oct4, Sox2, Klf4, and c-Myc (obtained from Addgene) were transfected into plat-E cells using polyJet transfection reagents (SignaGen). Passage 1 tail-tip fibroblasts (TTFs) were seeded on 6-well plates at a density of 1×10^5 cells/well for 24 h before retroviral infection. The retrovirus supernatants containing *Oct4*, *Sox2*, *Klf4*, and *c-Myc* were added equally to 6-well plates and incubated for 24 h with 4 μg/ml polybrene. The next day, the virus-containing medium was removed and replaced with fibroblast medium. On day 4, 5×10^4 virus-infected TTFs were seeded on mitomycin C-treated mouse embryonic fibroblast (MEF) feeders and incubated in ES-cell culture medium with leukemia inhibitory factor (LIF) until reprogramming was complete. After 4 passages, the homogenous $EGFP^+$ colonies were picked and evaluated for pluripotency.

2.2. RNA-Seq analysis

RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. Seven to ten million single-end RNA-Seq reads, of 101 basepairs in length, were generated using an Illumina sequencer and analyzed with CLC Genomics Workbench 4.9 (http://www.clcbio.com). Initially, the reads were trimmed according to quality score and base ambiguity. The processed reads were then mapped to the mouse RefSeq genomic sequences with annotations. The mapped reads of each gene were taken into account to calculate Reads per Kilobase of exon model per Million mapped reads (RPKM), a gene expression measure for RNA-Seq data. For non-specific matches, gene expression was first estimated based on the reads uniquely mapped to the gene, and then used as a weight for the distribution of non-specifically mapped reads. Consequently, 14480 of 34240 predicted genes had at least one mapped read and were detected in more than 3 of 6 samples. R-project was employed to perform the hierarchical clustering of these genes.

Additional methods are described in the Supplementary material.

3. Results and discussion

3.1. Cyclin D1 is induced during cell reprogramming and is co-expressed with neural progenitor markers but not with pluripotency markers

To identify the factors controlling the commitment of reprogramming cells to a pluripotent cell fate, TTFs isolated from 4-week-old mice carrying an Oct4-ΔPE-enhanced green fluorescent protein (Oct4-EGFP) reporter [18] were transduced with retroviruses carrying *Oct4*, *Sox2*, c-*Myc* and *Klf4*. The cells were then cultured on MEF feeders in ES-cell culture medium with LIF. After transduction for 19 days, cell clusters exhibiting a morphology similar to that of ES cells appeared in culture (Fig. 1A). However, very few cell clusters were found to express EGFP. Immunofluorescent staining revealed that the majority of $EGFP^-$ cell clusters were positive for Nestin (Fig. 1B), while $EGFP^+$ cells expressed Nanog (Fig. 1C). To further examine why $EGFP^-$ cells were not reprogrammed into pluripotent cells, the reprogramming cells were divided based on surface expression of EGFP and Stage-Specific Embryonic Antigen-1 (SSEA1) [19]. We distinguished three subpopulations using a FACSAria cell sorter: $EGFP^+/SSEA1^+$ (G^+/S^+), $EGFP^-/SSEA1^+$ (G^-/S^+), and $EGFP^-/SSEA1^-$ (G^-/S^-) (Supplementary Fig. 1A). The G^+/S^+ cell subpopulation was found to display high alkaline phosphatase (AP) activity and features of pluripotent stem cells, including expression of Nanog, SSEA1 and E-cadherin (Supplementary Fig. 1B–E). In contrast, the $EGFP^-$ cell subpopulations (G^-/S^+ and G^-/S^-) did not express EGFP or Nanog, although low levels of AP activity were present. RNA-seq analysis was performed on these three subpopulations together with TTFs, mouse TT2-ES cells, and mouse iPS cells (derived from 30 passages of G^+/S^+ cells in continuous culture). Hierarchical clustering analysis and RT-PCR analysis confirmed that a panel of pluripotency genes including *Nanog* and *Gdf3* were up-regulated in the G^+/S^+ cell population (Fig. 1D and E, Supplementary Fig. 2A and B). Both G^-/S^+ and G^-/S^- cell subpopulations expressed higher levels of *Ccnd1* (cyclinD1) together with some neural genes including *Pax6* and *Nestin*. Ingenuity Pathway Analysis (IPA) revealed that these neural genes are involved either in regulating the self-renewal of NPCs or in development of the central nervous system (Fig. 1D and E, Supplementary Fig. 2A–C). Immunofluorescent staining further confirmed that the majority of cyclin $D1^{hi}/EGFP^-$ cell clusters was positive for Nestin and Sox1 (Fig. 1F and G). These results suggest that reprogramming cells in the G^+/S^+ subpopulation were becoming pluripotent cells, while a large proportion of cyclin $D1^{hi}$ cells in the G^-/S^+ and G^-/S^- subpopulations were committed to neural progenitor fates.

3.2. Subpopulations of reprogramming cells exhibit distinct cell cycle structure

To explore the regulators that link cell cycle remodeling and cell fate commitment during Yamanaka factor-mediated fibroblast reprogramming, the expression patterns of cell-cycle regulators were examined in the different reprogramming cell populations after transduction with Yamanaka factors for 17 days. Immunofluorescent staining showed that $EGFP^-$ cell clusters that expressed Pax6 also displayed higher levels of Cdk6. In contrast, $EGFP^+$ cell clusters that were positive for Nanog expressed low levels of Cdk6 (Fig. 2A and B, arrowed cells). Western blot analysis further confirmed that cyclins A, B1, and H, as well as Cdk1 and Cdk7, were highly expressed in $EGFP^+$ cell subpopulations and in ES cells, while $EGFP^-$ cell subpopulations expressed high levels of cyclin D1, D2, and D3, and Cdk6 (Fig. 2C). We then investigated the percentage of cells in each phase of the cell-cycle using DAPI staining together with 5-ethynyl-2′-deoxyuridine (EdU) labeling (Fig. 2D). In contrast to TTFs, which have the majority of cells in G0/G1 phase, G^+/S^+ cells possessed cell-cycle characteristics similar to those of ES cells, with the majority of cells in S phase. The majority of G^-/S^- and G^-/S^+ cells were in G0/G1 phase.

KEGG mapping applied to the data obtained from RNA-seq analysis confirmed differences in the expression patterns of cell-cycle regulators among TTFs, G^-/S^-, G^-/S^+,G^+/S^+, iPS and ES cells (Supplementary Fig. 3A). G^+/S^+ cells expressed higher levels of S and G2/M phase regulators including *Ccne1*, *Ccna2* and *Ccnb1* (which are known to promote entry into S and G2/M phases, Supplementary Fig. 3C and D), while $EGFP^-$ cell subpopulations (G^-/S^- and G^-/S^+) expressed higher levels of G1 phase regulators including *Ccnd1*, *Cdk6*, *Cdkn1a*, and *Cdkn1b* (which are known to form kinase complexes regulating entry into G1 phase, Supplementary Fig. 3B). Further characterization showed that the majority of cyclin $D1^{hi}/EGFP^-$ cells were associated with expression of a panel of neural