A delayed h3k27me3 accumulation after DNA replication of embryonic stem cells opens chromatin for lineage specific transcription factors to bind and initiate differentiation

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INTRODUCTION

Pluripotent stem cells (PSCs) have been useful to generate differentiated progenies for cell replacement therapy, and disease models. The Parkinson's Disease (PD) field was arguably one of the first to have embraced the promise of stem cells. However, regardless of the differentiation protocols used, cultures and grafts continue to contain multiple cell types with midbrain dopamine (mDA) neural progenitors (NPs) and neurons representing only a fraction of total cells in the dish or graft. During cell differentiation, recruitment of transcription factors (TFs) to repressed genes in euchromatin is essential to activate new transcriptional programs, which is impeded by condensed H3K27me3-containing chromatin.

Here, using single-cell and gene-specific analyses, we tested the hypothesis that during the first hours of induction of differentiation of human embryonic stem cells (ESCs), accumulation of the repressive histone mark H3K27me3 is delayed after DNA replication, indicative of decondensed chromatin structure, potentially providing a critical 'window of opportunity' for recruitment of lineage-specific TFs to DNA. *If true, it may be possible to direct the differentiation of ESCs or iPSCs into homogeneous populations of any desired cell type needed to study, model and potentially treat different diseases including PD.*

MATERIALS AND METHODS

hESCs (H9 cells, Passage 35–50) were purchased from Wicell Research Institute and grown as feeder-free in mTesR1 medium (Stem Cell Technologies). Cell propagation was achieved through manual dissection and transfer of cut cell colonies once every 5 days. The differentiation was initiated by treating them with DMEM/F12 media (Life Tech) supplemented with 20% Knockout Serum Replacer™ (KOSR; Life Tech), 1% NEAA (Life Tech), 1x 2-mercaptoethanol (Life Tech), two TGF/BMP inhibitors SB431542 (SB, Tocris, 10 µM) and Dorsomorphin (DM, Tocris, 2 µM) and SHH (C24II) (SHH, R&D systems, 100 ng/ml), the SHH agonist Purmorphamine (Pur, Stemgent, 2 µM). Further differentiation down the mDA pathway is described in (Cai et al., 2009; Cai et al., 2013).

Chromatin Assembly Assay: hESCs were grown on chamber slides, pulse-labeled with 5 µM EdU and fixed at room temperature with 4% PFA in PBS for 15 min, washed with PBS, and permeabilized with 0.3% Triton for 15 min. Cells were subjected to Click-iT reaction (Invitrogen) with biotin-azide (Invitrogen) for 30 min. The PLA reactions (Olink) between the anti-biotin antibody and antibodies to other proteins were performed as described previously (Petruk et al., 2013; Petruk et al., 2012). Following PLA, cells were immunostained with anti-biotin Alexa Fluor 488 antibody to control the specificity of CAA. The results of CAA experiments were quantified by counting the number of PLA signals per EdU-labeled nuclei in 50 cells of each of the three independent experiments.

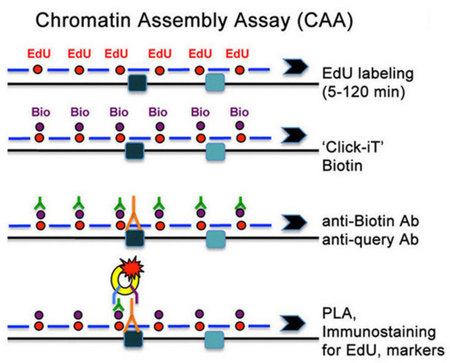


Figure 1. A scheme of CAA. Bio: Biotin, PLA: proximity ligation assay.

RESULTS

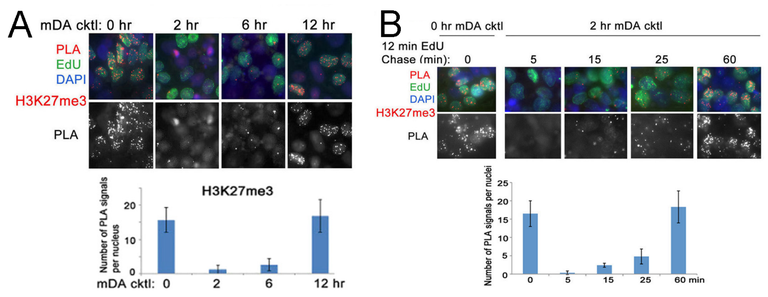


Figure 2. Accumulation of H3K27me3 in Single Cells following DNA Replication during Induction of Differentiation of hESCs. (A) hESCs were grown for 4 days in mTeSR1 medium and then induced with the mDA cocktail to the mDA lineage for the indicated times. Cells were labeled with EdU for 15 min. (B) hESCs were grown for 4 days in mTeSR1 medium and then induced for 2 hr with the mDA cocktail. Cells were labeled with EdU for 12 min and chased for the indicated times. CAA was performed for H3K27me3, followed by immunostaining for biotin (EdU). PLA, red; biotin, green; DAPI, blue. PLA only is shown in black and white. Quantification of the results of three independent CAA experiments is shown below

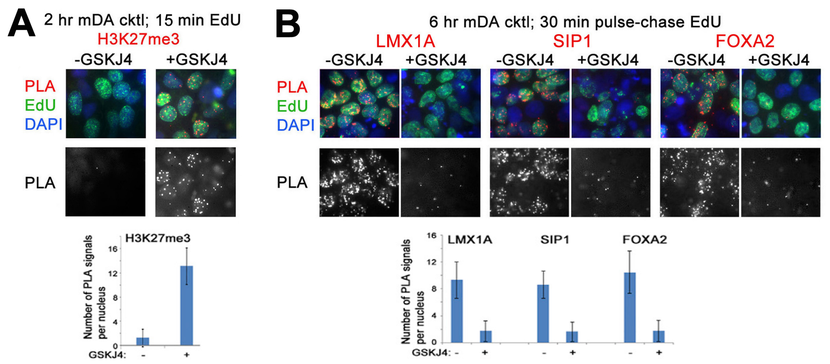


Figure 5. Lack of H3K27me3 in Post-replicative Chromatin Is Essential for the Association of Lineage-Specific TFs with DNA in hESCs. (A) hESCs were grown in mTeSR1 medium for 4 days and then for 4 hr in the absence and presence of 10 µM GSKJ4, an H3K27 demethylase inhibitor and induced to the mDA lineage for 2 hr. Cells were labeled with EdU for 15 min. CAA was performed for H3K27me3, followed by immunostaining for biotin (green). PLA only is shown in black and white. (B) hESCs were grown and induced to the mDA lineage for 6 hr with and without GSKJ4. Cells were pulse-labeled with 5 µM EdU for 15 min and chased for 15 min. CAA was performed for LMX1A, SIP1, and FOXA2, followed by immunostaining for biotin (green). PLA only is shown in black and white.

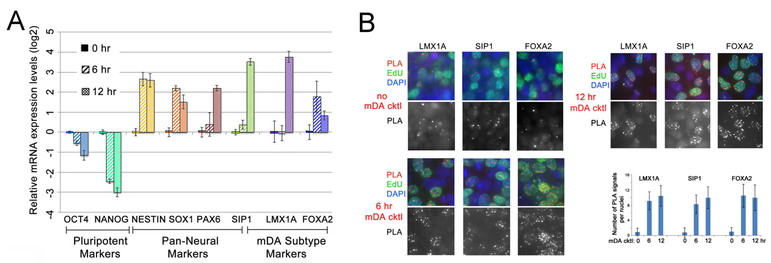


Figure 3. Induction of Expression and Association with DNA of Lineage-Specific TFs during mDA Differentiation of hESCs. (A) qRT-PCR gene expression analysis of undifferentiated (0 hr, control) hESCs and hESCs induced to the mDA lineage for 6 and 12 hr. (B) Undifferentiated hESCs and hESCs induced to the mDA lineage for 6, 12 hr were labeled with EdU for 15 min and then chased for 15 min. CAA was performed for LMX1A, FOXA2, and SIP1, followed by immunostaining for biotin (green). PLA only is shown in black and white. Quantification of the results of three independent CAA experiments is also shown.

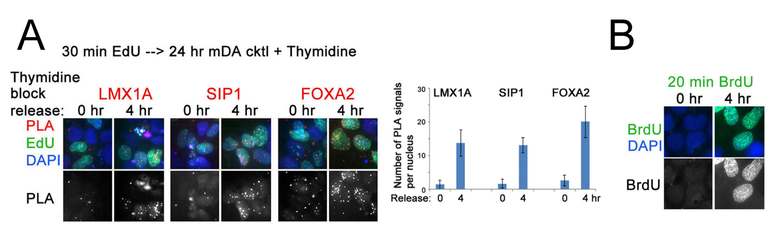


Figure 4. Lineage-Specific TFs Associate with DNA Shortly after DNA Replication (A) hESCs were grown for 4 days in mTeSR1 medium and labeled with EdU for 30 min. Cells were induced to the mDA lineage and grown for 24 hr in the presence of thymidine. The thymidine block was removed for 0 hr (left) and 4 hr (right). CAA was performed for LMX1A, FOXA2, and SIP1, followed by immunostaining for biotin (green). PLA only is shown in black and white. Quantification of the results of three independent CAA experiments is shown on the right. (B) Thymidine block release was monitored by 20-min incorporation of BrdU (green).

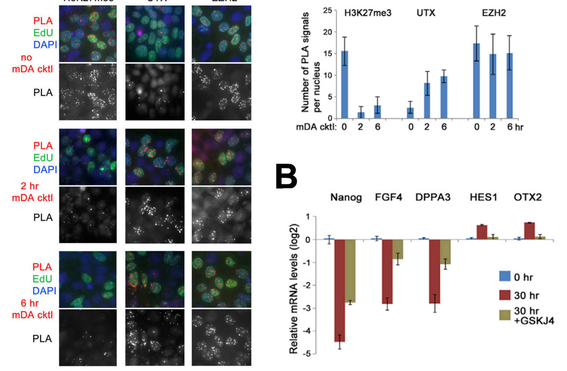
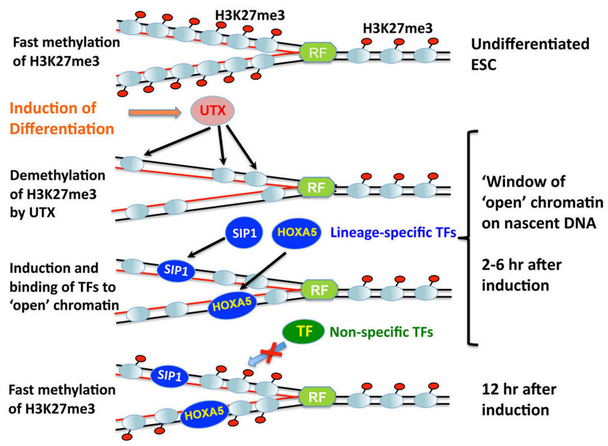


Figure 6. Association of H3K27me3 HMT and KDMs with DNA during Induction of Differentiation of hESCs. (A) hESCs were grown in mTeSR1 medium for 4 days and then induced to the mDA lineage for 2 and 6 hr. Cells were labeled with EdU for 15 min, and CAA was performed for H3K27me3, UTX, and EZH2, followed by immunostaining for biotin (green). PLA only is shown in black and white. (B) qRT-PCR analysis of the expression of pluripotency and differentiation markers in hESCs. Undifferentiated cells (0 hr) were induced with mDA for 30 hr, in the absence and presence of 10 µM GSKJ4. The following markers were tested: NANOG, FGF4, and DPPA3 for pluripotency, HES1 and OTX2 for differentiation.

SUMMARY



Using CAA, the single cell analysis of histones and other key proteins associated with nascent DNA, we demonstrated a critical role for chromatin that is assembled shortly after DNA replication during the differentiation of pluripotent stem cells. The state of chromatin may be one of the earliest and most reliable indicators of the state of differentiation in cells, making it possible to identify bona fide undifferentiated stem cells ideal for directing the differentiation of homogenous mDA neurons for studies of PD in vitro and in vivo.

ACKNOWLEDGEMENTS

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