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Nuclear and chromatin reorganization in the *MHC-Oct3/4* locus at developmental phases of embryonic stem cell differentiation

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Abstract

Epigenetic gene control is involved in mechanisms of development. Little is known about the cooperation of nuclear and chromatin events in programmed differentiation from mouse embryonic stem cells (ESC). To address this, Oct3/4-positive ESC and differentiated progenies, Sox1-positive neural precursor cells (NPC) and post-mitotic neurons (PMN), were isolated using a stage-selected culture system. We first investigated global nuclear organization at the each stage. Chromocenter preexists in ESC, disperses in NPC and becomes integrated into large heterochromatic foci in PMN, while the formation of PML bodies markedly decreases in neural differentiation. We next focused on the gene-dense *MHC-Oct3/4* region. *Oct3/4* gene is expressed preferentially adjacent to PML bodies in ESC and are repressed in the absence of chromocenter association in NPC and PMN. Histone deacetylation in NPC, demethylation of lysine 4 of histone H3 (H3K4), tri-methylation of H3K27, and CpG methylation in PMN are targeted for the *Oct3/4* promoter within the region. Interestingly, di-methyl H3K4 mark is present in *Oct3/4* promoter in NPC as well as ESC. These findings provide insights into the molecular basis of global nuclear reorganization and euchromatic gene silencing in differentiation through the spatiotemporal order of epigenetic controls.

Keywords: Nuclear structure; Histone modification; DNA methylation; Differentiation; Embryonic stem cells

Introduction

Cell differentiation is the programmed changes of stem cells and progenitors, through epigenetic control of chromatin and gene expression in nucleus (Francastel et al., 2000). It has been suggested that the global distribution of acetylated histone H3 produces a key feature of undifferentiated stem cells (Kimura et al., 2004; Lee et al., 2004). In this context, differentiation originating from stem cells may be a process of converting transcriptionally active chromatin into stably silenced chromatin (Gasser, 2002). Genome-wide gene expression analysis revealed that mouse stem cells such as embryonic stem cells (ESC) and neural stem cells express approximately 40–60% of the total genes in a genome, including a number of house-keeping and tissue-specific genes. In contrast, most differentiated cells express only 10–20% of the total genes (Abeyta et al., 2004). Indeed, terminally differentiated erythroblasts have a greater amount of condensed chromatin and a smaller sized nuclear volume, compared to proerythroblasts (Francastel et al., 2000).

Morphologically, the chromatin structure is divided into two heritable forms in interphase nuclei, transcriptionally active euchromatin and inactive heterochromatin. Recent studies have emphasized the relationship between higher ordered chromatin structure and specific DNA/histone modifications (Jenuwein and Allis, 2001). In euchromatic regions, histones H3/H4 are hyperacetylated on lysine residues at the amino-terminal tail of proteins. Conversely, heterochromatic regions are characterized by low levels of acetylation. Further, methylation of specific lysine residues of H3 provides complex information to the chromatin structure (Lund and van Lohuizen, 2004). Methylation of lysine 4 of H3 (methyl H3K4) is generally observed in euchromatic regions, while both methyl H3K9 and methyl

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H3K27, in combination with DNA methylation at CpG dinucleotide, are found in heterochromatic regions. In addition, modified histones and DNA are also recognized by specific binding of chromatin factors (Lund and van Lohuizen, 2004). Thus, epigenetic marks represent the state of chromatin and transcriptional activity of a genomic region, which play an essential role in developmentally coordinated gene regulation and differentiation of stem cells. For instance, post-implantation stage development and ESC differentiation is severely impaired in the mutant mice lacking the histone methyltransferase or DNA methyltransferase, although these activities are dispensable for ESC self-renewal (En Li et al., 1992; Okano et al., 1999; Tachibana et al., 2002). The methylation of histone and DNA is crucial epigenetic marks for establishment of cellular identity during differentiation, but the molecular basis of these findings remains unclear.

Heterochromatin structure is formed by either *cis*- or *trans*-acting mechanisms. The *cis*-spreading mechanism of heterochromatin assembly utilizes the SET domain-containing Suv39h1-mediated methylation of H3K9, followed by recruitment of chromodomain protein HP1. HP1 then recruits Suv39h1 to methylate neighboring histones, resulting in the spread of heterochromatin in the pericentromeric region (Bannister et al., 2001). In the non-pericentromeric region, unique *cis*-elements, termed PRE (polycomb responsive elements) are implicated in developmentally regulated heterochromatin formation in *Drosophila*, where another SET domain protein E(z) produces H3K27 methylation that is recognized by Pc in the polycomb protein complex (Cao et al., 2002). On the other hand, intranuclear positioning of a specific gene locus relative to constitutive heterochromatin has become one of the mechanisms for *trans*-acting chromatin regulation. DNA-binding factor Ikaros is reported to bind the promoter region of the *CD2* or *CD19* genes as well as pericentromeric repeat DNA. During thymocyte development, Ikaros and its target genes are repositioned to the proximity of heterochromatin foci in a lineage-specific manner (Brown et al., 1997). In addition, silenced allele of the immunoglobulin locus preferentially locates at the nuclear periphery in non-B lymphocytes, where stable silencing is mediated by lamina-associated heterochromatin (Kosak et al., 2002). Similarly, *cis*- and *trans*-types of chromatin regulation are likely to occur in euchromatic regions. In yeast, Sas2p acetyltransferase-dependent H4K16 acetylation and the subsequent binding of bromodomain protein Bdf1 cause *cis*-spreading of euchromatin (Ladurner et al., 2003). In mammals, several transcribed euchromatic regions have been reported to localize around PML bodies containing histone acetyltransferase and other proteins (Wang et al., 2004; Ching et al., 2005). Thus, compartmentalization of inactive or active gene loci into the specific subnuclear positions may be a common mechanism for gene regulation (Francastel et al., 2000).

Although the significance of epigenetic regulation in cell differentiation has been accepted, it remains unclear how nuclear substructure and chromatin in specific chromosomal region are cooperatively controlled in the process of differentiation. The POU transcriptional factor *Oct3/4* (*pou5f1*) gene plays a critical role in mouse embryonic development and in adult tissue (Nichols et al., 1998; Ramos-Mejia et al., 2005; Hochedlinger et al., 2005). It is known that *Oct3/4* expression is restricted to pluripotent cell lineages including germ cells, early-stage embryos and the derivative embryonic stem cells (Nichols et al., 1998). During mouse embryogenesis or in vitro differentiation of ESC, downregulation of the *Oct3/4* gene strongly correlates with loss of pluripotency. Further, the abnormal expression of *Oct3/4* in non-pluripotent cells results in various developmental abnormalities, including defects in brain patterning, hyperproliferation of epithelial dysplasia and eventually causes tumors in multiple tissues (Ramos-Mejia et al., 2005; Hochedlinger et al., 2005). Thus, developmental repression of *Oct3/4* must be irreversible to prevent unscheduled cell regulation and potential oncogenic activities. It is of particular interest that the *Oct3/4* locus is located in the most gene-rich euchromatic region, the major histocompatibility complex (*MHC*) gene cluster in mouse chromosome 17B1 (Nordhoff et al., 2001; Horton et al., 2004). The human *MHC-Oct3/4* locus conserved in chromosome 6p21.3 contains 421 genes 7.6-Mbp long; in other words, the average gene density is one gene per 18 kbp. This gene richness is exceptionally high, compared to the predicted average from the whole human genome (one gene per 135 kbp). There must be a unique mechanism to selectively repress the *Oct3/4* gene within the gene-rich euchromatic region, without affecting the chromatin status of the adjacent genes. In the present study, we established a lineage- and stage-specific neural differentiation of mouse ESC in order to investigate global nuclear reorganization and epigenetic remodeling in the *MHC-Oct3/4* locus during differentiation. Our findings provide insight into the molecular basis of global and local remodeling in differentiating nuclei, and the developmental significance of epigenetic marks in the *Oct3/4* locus.

Materials and methods

Cell culture and differentiation

Mouse ESC (EB3 or 46C) were maintained on feeder-free 0.1% gelatin-coated dishes in knockout-DMEM (KO-DMEM, Gibco) supplemented with 0.3% fetal bovine serum (FBS) (Hyclone), 15% knockout-serum replacement (KSR, Gibco), 2 mM Glutamate, 0.1 mM 2-mercaptoethanol, 1× non-essential amino acids (NEAA, Gibco) and leukemia inhibitory factor (LIF). EB3 [Oct3/4$^{+/\text{ires-}bsd\text{-}pA}$] and 46C [Sox1$^{+/gfp\text{-ires-}pac\text{-}pA}$] cells were established from parental E14tg2a cells (Nichols et al., 1998; Niwa et al., 2002; Aubert et al., 2003). EB3 was cultured in a medium containing 5 μg/ml blastocidin S to eliminate spontaneously differentiated cells. To induce neural differentiation of ESC, PA6 feeder cells were grown in DMEM/F12 supplemented with 15% FBS and fixed with 4% paraformaldehyde in PBS for 20 min (Kawasaki et al., 2000). EB3 and 46C cells (1×10^3 cells/cm^2) were seeded onto the fixed PA6 cells in KO-DMEM containing 15% KSR, 2 mM glutamate, 0.1 mM 2-mercaptoethanol, and 1× NEAA. To obtain homogeneous populations of neural cells, differentiating 46C cells were replaced to poly-L-ornithine (Sigma) and bovine plasma fibronectin (Sigma) coated dishes in N2B27 medium supplemented with 20 μg/ml recombinant human bFGF2 (Invitrogen) at day 6. Sox1-positive neural precursor cells were selected under 0.5 μg/ml puromycin (Sigma) at days 8–12. To isolate post-mitotic neurons, cells were cultured in N2B27 medium containing 200 μM L-ascorbic acid (sigma) at day 6. At day 9, the addition of 1 μM AraC (Sigma) eliminated mitotic cells for a further 3 days.