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Protein and mRNA levels of IgM H- and L-chains artificially and excessively accumulated in HDAC2-deficient DT40 mutants are gradually reduced via a lot of generations during continuous cultivation**

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Abstract

We analyzed HDAC2-deficient DT40 mutant cells, HDAC2(-/-), which were cultivated for varying periods, by 2D-PAGE, Western blotting, RT-PCR and immuno-electron microscopy. Protein and mRNA levels of IgM H- and L-chains were increased at the early stage of cultivation and thereafter until the late stage reduced to comparable levels in DT40 cells. The mRNA level of PCAF was increased from the early to late stages, and those of HDAC7 and HDAC9 also changed. The mRNA level of EBF1 was almost completely decreased at the early stage and thereafter remained unchanged, but that of E2A was slightly increased at the early stage and thereafter remained unchanged. The mRNA level of Pax5, which down-regulates gene expressions of IgM H- and L-chains, was reduced at the early stage and thereafter increased via the middle to late stages in anti-parallel with those of these two immunoglobulin proteins. Bulk acetylation levels of Lys-9, Lys-14, Lys-18, Lys-23 and Lys-27 residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) were increased during cultivation. Chromatin immuno-precipitation assay suggested that acetylation levels of K9/H3 within some chromatin segments surrounding proximal ~2.0 kb 5'-upstream region of the Pax5 gene were decreased at the early stage in HDAC2(-/-) and thereafter at the late stage increased to comparable levels in DT40 cells.

These results revealed not only that the HDAC2-deficiency induces dramatic increases in mRNA and protein levels of IgM H- and L-chains, and thereafter these accumulated mRNAs and proteins are gradually reduced during cultivation, but also that alterations in gene expressions of IgM H- and L-chains may be coupled with changes in those of Pax5, EBF1, PCAF, HDAC7 and HDAC9. In addition, the changing pattern in acetylation levels of K9/H3 within proximal 5'-upstream chromatin region of the Pax5 gene in HDAC2(-/-) should qualitatively agree with that in its transcription level.

Keywords

HDAC2-deficient DT40 mutants; Continuous cultivation; Decreases in IgM H/L chain protein/mRNA levels; Changes in mRNA levels of transcription factors; Gene targeting techniques; ChIP assay; Changes in acetylation levels of K9/H3 surrounding Pax5 5'-upstream chromatin region

Introduction

The modulation of chromatin topology with epigenetic modifications is one of the most important manners for expression of cell functions in eukaryotes. Alterations in chromatin structure are preferentially involved in controls of gene expression/transcription and DNA replication, repair and recombination and so on [1-3]. Naturally, such alterations are concerned in regulations of lymphocyte development and differentiation [4-7]. Moreover, regulation of the lymphocyte development requires a number of transcription factors, including Ikaros, E2A, PU.1, GATA-3, Pax5, EBF and so on, and transcriptional regulation of IgM H-chain requires USF, TFEB, Ig/EBP, NF-IL6, OCA-B and others as

promoter binding proteins and Ig/EBP, NF-IL6, YY-1, E2A, PU.1 and others as intron enhancer binding proteins [8-13]. On the other hand, of various epigenetic modifications of chromatin structure with acetyl, methyl and phosphate groups and so on, acetylation and deacetylation of particular Lys residues (K) of core histones (H2A, H2B, H3 and H4) may be major ones [14-21]. They are cooperatively controlled in combination of particular members of chromatin modifying enzymes, i.e., histone acetyltransferase(s) (HATs) and histone deacetylase(s) (HDACs) [22, 23]. To clarify in vivo roles of individual members of HATs and HDACs in expressions of cell functions, we have systematically generated a number of homozygous (or conditional) chicken DT40 mutant cell lines lacking appropriate genes [24-33] by gene targeting techniques [34-39]. Our previous analysis of HDAC2-deficient DT40 mutant cells, HDAC2(-/-), revealed that HDAC2 controls the amount of IgM H-chain at the steps of its gene expression plus pre-mRNA alternative processing [24] and down-regulates IgM L-chain gene promoter activity [27]. Moreover, the HDAC2-deficiency decreases gene expressions of HDAC7, Pax5, Aiolos, Ikaros plus EBF1, increases those of HDAC4, HDAC9, PCAF plus E2A, and changes bulk acetylation levels of several particular Lys residues of core histones H2A, H2B, H3 and H4 [30].

Subsequently, to know individual roles of these altered transcription factors in regulations of IgM H- and L-chain gene expressions, we generated homozygous DT40 mutant cells; EBF1(-/-), Aiolos(-/-), E2A(-/-) and Helios(-/-), devoid of two alleles of EBF1, Aiolos, E2A and Helios genes, respectively [30]. In addition, we generated Pax5-deficient mutant cells, Pax5(-), devoid of the Pax5 gene located on Z chromosome that is monosomy in chickens (USCS Genome Browser data base) [40], but could generate only Ikaros-down mutant cells, Ikaros(-/-/+), devoid of two alleles of the gene located on chromosome 2 that is trisomy, since it should be very important or essential for DT40 cells (data will be shown elsewhere). Analyses of these resultant mutants revealed that Pax5, EBF1, Aiolos plus Ikaros down-regulate IgM H- and L-chain gene expressions, and E2A up-regulates gene expressions of these two immunoglobulin proteins [30]. Thus, these results indicated that HDAC2 indirectly controls gene expressions of IgM H- and L-chains through opposite regulations of gene expressions of Pax5, EBF1, Aiolos plus Ikaros, and E2A [30, 31].

In this study, we analyzed HDAC2(-/-) at different cultivation periods by 2D-PAGE, Western blotting, immuno-electron microscopy and RT-PCR. During cultivation of HDAC2(-/-), protein and mRNA levels of IgM H- and L-chains were dramatically and considerably elevated at the early stage, and thereafter gradually reduced and finally at the late stage reached to comparable levels in DT40 cells. In addition, mRNA levels of HDAC7, HDAC9 and PCAF were gradually elevated, and those of Pax5, Aiolos, E2A and EBF1 were certainly altered in different patterns. Further, we cloned ~4.9 kb 5'-upstream region of the Pax5 gene from DT40 genomic DNA by our original gene walking techniques. Chromatin immuno-precipitation (ChIP) assay suggested that acetylation levels of Lys-9 residues of histone H3 (K9/H3) within the proximal ~2.0 kb 5'-upstream chromatin region of the Pax5 gene were

slightly decreased at the early stage in HDAC2(-/-) and at the late stage increased to comparable levels in DT40 cells. We discussed on the relationship among these changing patterns in gene expressions of IgM H- and L-chains, particular transcription factors and chromatin modifying enzymes, and acetylation levels of K9/H3 within the proximal ~2.0 kb 5'-upstream chromatin region of the Pax5 gene in HDAC2(-/-) mutants during continuous cultivation.

Methods

Cell cultures

Generation of HDAC2(-/-) DT40 mutants was described in our previous report [24]. All positive mutant clones had been picked up on 96-well plates at ~10 days after knockout of two HDAC2 alleles, cultivated for another several days to be ~10^6 cells/ml and stocked at -80°C until use. HDAC2(-/-) mutant clones were continuously cultivated as described [24, 30] and collected at varying interval periods, including the early (E), middle (M) and late (L) stages of cultivation. The cultivation periods and/or stages were practically counted from the first day of cultivation from the stock at -80°C.

2D-PAGE

Whole cellular proteins were prepared from HDAC2(-/-) mutant clone (cl.33-12) at the early (E; ~10 days), middle (M; ~30 days) and late (L; ~60 days) stages of cultivation and DT40 (W), all of which were exponentially growing, and separated on 2D-PAGE using an automated apparatus Multiphor II (Amersham Pharmacia Biotech.), followed by staining with SYPRO Red (Molecular Dynamics, Inc.) as described [24, 37-39, 41].

Western blotting

Whole cellular proteins were prepared from HDAC2(-/-) mutant clones (cl.33-28, cl.33-30 and cl.45-28) at different cultivation periods (up to ~56 days) and DT40 (W) as described [25, 30]. Briefly, cells (1 x 10^7) were treated with 10% trichloroacetic acid (TCA) and lysed in 100 μl of SDS buffer. Aliquots (10 μl) of 1:50 dilutions of the resultant cell extracts were subjected to 12% SDS-PAGE and electro-transferred to membranes, and proteins were detected with rabbit anti-chicken IgM L-chain antiserum A30-100A (Bethyl Laboratories Inc., TX, USA) that cross-reacts with IgM H-chain as described [28, 30]. Data analyses were carried out by Multi Gauge Ver3.X software using a luminescent image analyzer LAS-1000plus (FUJIFILM) [28, 30]. The relative amounts of IgM H-chain, and large (high) and small (low) forms of IgM L-chain were measured at indicated periods.

Immuno-electron microscopy

Post-embedding immunocytochemistry was carried out as described [42]. Exponentially growing

DT40 (W) and HDAC2(-/-) mutant clone (cl.33-28) at the early (E; ~10 days) and late (L; ~60 days) cultivation stages were collected by a light centrifugation, and the resultant cell pellets were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB) for 30 min. After washing with PB, the samples were post-fixed with 1% osmium tetroxide in PB for 60 min, washed with PB, dehydrated in ethanol and embedded in Epon. Ultrathin sections were picked up on 200-mesh gold grids coated with Formvar film and treated with 5% sodium meta-periodate in distilled water for 30 min. After rinsing in distilled water, the sections were treated with 5% normal horse serum (NHS) and 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 10 min to block nonspecific binding and incubated with goat anti-chicken IgM H-chain antibody A30-102A (Bethyl Laboratories Inc., TX, USA; diluted 1:1500 with 5% NHS, 1% BSA in PBS) at room temperature for 60 min. After rinsing in PBS, the sections were incubated with biotinylated horse anti-goat IgG (Vector Laboratories, Burlingame, CA, USA; diluted 1:200 with 1% BSA in PBS) for 40 min. After washing with PBS, the sections were incubated with 8 nm colloidal gold conjugated anti-biotin antibody (Vector Laboratories, Burlingame, CA, USA; diluted with 1% BSA in PBS) for 30 min. After washing with distilled water and drying, the sections were contrasted with 2% uranyl acetate in 70% methanol and Reynolds' lead citrate and observed in a JEOL 1200EX transmission electron microscope operating at 80 kV (JEOL, Tokyo, Japan). As control, primary antibody was omitted or replaced by normal goat serum.

RT-PCR

Total RNAs were isolated from three independent HDAC2(-/-) mutant clones (cl.33-28, cl.33-30 and cl.45-28) at the early (E; ~20 days), middle (M; ~40 days) and late (L; ~60 days) stages of cultivation and DT40 (W) as described [24, 30, 43]. RT-PCR was carried out using appropriate sense and anti-sense primers used in our previous papers [28, 30] and listed in Table I. HDAC2 and β-actin were used as negative and positive controls. RT-PCR products were subjected to 15% agarose gel electrophoresis. Data analyses were carried out as described above. Nucleotide sequences of all amplified products were confirmed by the PCR sequencing protocol as described [28, 30].

Immunoblotting

Whole cellular proteins were prepared from three independent HDAC2(-/-) mutant clones (cl.33-28, cl.33-30 and cl.45-28) at the early (E; ~20 days), middle (M; ~40 days) and late (L; ~60 days) stages of cultivation and DT40 (W) (three different cultures) as described [30]. Briefly, cells (1×10^7) were treated with 10% TCA and lysed in 100 μl of SDS buffer. Aliquots (10 μl) of the resultant cell extracts were subjected to 15% SDS-PAGE and electro-transferred to membranes. Bulk acetylation levels of particular Lys residues (K) of core histones H3, H4, H2A and H2B (indicated by appropriate designations) and the bulk methylation level of K9/H3 were measured by immunoblotting using

site-specific anti-acetylated and anti-methylated histone antibodies as described [30]. Antibodies used were: anti-AcK9H3 (#06-942), anti-MeK9H3 (#07-212), anti-AcK14H3 (#06-911), anti-AcK18H3 (#07-354), anti-AcK23H3 (#07-355), anti-AcK27H3 (#07-360), anti-AcK8H4 (#06-760), anti-AcK12H4 (#07-323), anti-AcK16H4 (#07-329), anti-AcK5H2A (#07-290), anti-AcK7H2A (#07-386), anti-AcK9H2A (#07-289), anti-AcK5H2B (#07-382), anti-AcK12H2B (#07-336), anti-AcK15H2B (#07-343), anti-AcK16H2B (#07-341) and anti-AcK20H2B (#07-347) from Upstate and anti-AcK5H4 (AB3064) from CHEMICON.

Cloning of ~4.9 kb 5'-upstream fragment of chicken Pax5 gene

Although the chicken Pax5 gene has been reported to be located on Z chromosome that is monosomy and to consist of 10 exons at the present time, the detailed information, including nucleotide sequences of the 5'-upstream region plus 5'-untranslational region of its first exon, was not available from USCS Genome Browser database until we had started this study. Only nucleotide sequences of chicken Pax5 cDNA [8] and 5'-upstream genomic DNA sequences of exon 1 up to position -109 (GenBank accession number: NM_204424) had been reported. To clone and identify the ~4.9 kb 5'-upstream fragment of the Pax5 gene, we first screened the DT40 genomic DNA library in a λFIXII [24] by PCR using sense primer (5'-GCACACTACGGACAAACTTTTCC-3') and antisense primer (5'-CGGGGCCGCGTACATCTTCTCCAAATCCAT-3'), which were designed to amplify a part of exon 1 of the gene. However, no positive clones were yielded.

Therefore, we directly cloned the ~4.9 kb 5'-upstream region of the Pax5 gene from DT40 genomic DNA by our original gene walking techniques, including Southern blotting [44], sub-cloning and colony hybridization (details will be shown later). First, the genomic DNA was digested with appropriate restriction enzymes, recognizing particular positions in multiple cloning site of pBluescript II SK(+) vector, and hybridized with the PCR amplicon (as probe), corresponding to the 85 bp fragment of exon 1 (positions -55 ~ +30), which was obtained from the genomic DNA using the above-mentioned primers. Among positive fragments obtained, the ~2.1 kb XbaI/ClaI fragment was sub-cloned into the multiple cloning site of pBluescript II SK(+) in frame as mentioned below. Enough amounts of the genomic DNA (200 μg) were digested with XbaI and ClaI at 37℃ for 18 hr and separated in 0.8% agarose gel, and the gel area containing the desired DNA fragment of ~2.1 kb was excised. The ~2.1 kb DNA fragments were extracted using the filter cartridge SUPEC™-01 (TaKaRa) following the manufacture's protocol and recovered by phenol/chloroform extraction and ethanol precipitation. The purified DNAs were dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and inserted into the XbaI/ClaI site of pBluescript II SK(+) in molar ratio of 1 to 10 (for vector to insert) using DNA Ligation Kit Ver.1 (TaKaRa) at 16℃ for 21 hr. The ligated plasmid was introduced into XL-1 blue MRF Competent cells, and then the cells were plated onto LB/amp plates, incubated at 37℃ for overnight, cooled at 4℃ for 1

hr, and subjected to colony hybridization with the 85 bp PCR amplicon as probe. Our screening yielded one positive colony. Then, we sequenced the cloned ~2.1 kb DNA fragment by the dye terminator method using Big Dye Terminator V3.1 Sequencing Standard kit (Applied Biosystems).

As the next step, using the similar techniques, we cloned and identified nucleotide sequences of more additional ~3.0 kb 5'-upstream region of the Pax5 gene as follows. Based on the 5'-upstream genomic sequences mentioned above, sense 5'-TGCCATTTTCAAACACGCGC-3' and antisense 5'-GGAAAAGTTTGTCCGTAGTGTGC-3' primers were constructed. The 1626 bp PCR amplified product (corresponding to positions -1656 ~ -32) was obtained from the genomic DNA using these primers and digested with BssHII. After separation in 0.8% agarose gel, the band of desired 263 bp fragment was excised and purified using Wizard SV Gel and PCR Clean-Up System (Promega).

Successively, resultant 263 bp DNA fragment, corresponding to the region from positions -1656 ~ -1394, was used as probe for subsequent hybridization to obtain more additional 5'-upstream genomic fragments cut with appropriate restriction enzymes. Among positive fragments obtained, the ~4.9 kb BamHI/ClaI fragment was inserted into the BamHI/ClaI site of pBluescript II SK(+) in molar ratio of 1 to 5 (for vector to insert) and introduced into XL-1 blue MRF Competent cells. Then, the cells were plated onto LB/amp plates and subjected to colony hybridization. Finally, we isolated three positive clones, possessing the ~4.9 kb 5'-upstream fragment from nucleotide number +241, and sequenced the isolated DNA fragment as described above.

Colony hybridization

We carried out colony hybridization on Hybond-N+ nylon membrane filter following the manufacture's protocol (Amersham Biosciences). In brief, the membrane was laid on the surface of agar plate for 45 sec until it became thoroughly wetted, peeled carefully, and then its colony side was placed in upper direction onto a 10%(w/v) SDS saturated 3MMChr paper (Whatman) for 3 min. DNA was denatured and fixed to the membrane by successive treatments with denaturization buffer (0.5 M NaOH, 1.5 M NaCl) for 7 min and neutralization buffer (1.5 M NaCl, 0.5 M Tris-HCl; pH 7.4) for 3 min twice. Finally, the membrane was vigorously washed in 2X SSC (0.3 M NaCl, 30 mM $C_6H_5O_7Na_3$-$2H_2O$; pH7.0) and its surface was gently scraped using KIMWIPE soaked in 2X SSC to remove cell debris. DNA was fixed to the membrane by a UV cross-linking procedure and hybridized with ^{32}P-labeled probe by means of conventional methods.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was done with ChIP assay kit (Millipore) according to manufacturer's instructions. Briefly, DT40 (W) and HDAC2(-/-) mutant clones (cl.33-28, cl.33-30 and cl.45-28) (1 x 10^6 cells) at the early (E; ~20 days) and late (L; ~60 days) stages of cultivation were

cross-linked by the addition of 37% formaldehyde to a final concentration of 1% and incubated at 37℃ for 10 min, and the cross-linking was stopped with the addition of one-tenth volume of 1.25 M glycine to a final concentration of 0.125 M at 37℃ for 5 min. After removing medium, cells were washed with ice-cold PBS twice and re-suspended in 200 μl of SDS Lysis Buffer freshly supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) (Wako, Osaka, Japan) and 1 μg/ml approtinin (Sigma, St Louis, MO, USA), followed by sonication to solubilize and to shear cross-linked DNA. At this step, we used a Bioruptor UCD-250 (Cosmo Bio) at power L for ten 30-s pulses (30-s pause between pulses) at 4℃ to shear chromatin DNA into fragments of approximately 200 ~ 1000 bp. The samples were centrifuged at 13,000 rpm at 4℃ for 10 min to remove cell debris from the crude chromatin lysate. Sonicated cell supernatant was diluted to 1/10-fold by Chip Dilution Buffer. For input, a portion (40 μl: 2%) of the diluted cell supernatant was kept to quantify the amount of DNA present in each of different samples at PCR step. Antibody (2 μl) was added to 2 ml of the supernatant fraction and incubated at 4℃ for overnight. Incubation with 60 μl of Protein Agarose/Salmon Sperm DNA was continued for 1 hr, followed by centrifugation at 1,000 rpm at 4℃ for 1 min. The immuno-precipitated complexes were sequentially washed with low-salt solution, high-salt solution, LiCl solution and TE (pH 8.0) twice. The antibody/histone/DNA complexes were eluted from Protein Agarose beads by adding 250 μl of the elution buffer (1% SDS, 0.1% $NaHCO_3$) twice, and the cross-linking was reversed at 65℃ for 6.5 hr by the addition of 5 M NaCl to a final concentration of 0.2 M. Samples were treated with proteinase K at 37℃ for 1 hr to remove proteins. DNA was recovered by phenol/chloroform extraction and ethanol precipitation using 40 μg glycogen (NAKARAI TESQUE, INC.) as inner carrier. Anti-AcK9H3 (#07-352) (Millipore) was used for pulldown. Recovered DNA fragments were subjected to PCR for 25 ~ 35 cycles, using appropriate primers (see Table II) specific for segments A to Q of approximately 150 ~ 250 bp, corresponding to proximal 5'-upstream region and first exon (including the start codon) of the Pax5 gene. PCR was carried out using the same master reaction mixture in 25-μl scale. Immuno-precipitated chromatin treated with non-immune rabbit serum (Vector Laboratories) (as negative control) produced no bands following PCR (data not shown).

Results

IgM H- and L-chains artificially accumulated in HDAC2(-/-) are gradually reduced during continuous cultivation

To know changing patterns in cellular protein levels of HDAC2(-/-) mutants during cultivation, we first analyzed total cellular proteins prepared from clone cl.33-12 of HDAC2(-/-) mutants at the early (E; ~10 days), middle (M; ~30 days) and late (L; ~60 days) stages of cultivation and DT40 cells (W) as control by 2D-PAGE (Fig. 1). The amounts of IgM H-chain and L-chain (detected as two spots), whose amino acid sequences had been determined in our previous paper [24], were dramatically and certainly

increased at the early cultivation stage in mutant clone cl.33-12 compared with those in DT40 cells. Surprisingly, the elevated amounts of these two immunoglobulin proteins in clone cl.33-12 were gradually reduced via the middle to late cultivation stages to almost similar levels in DT40 cells, whereas changes in cellular levels of most of other major proteins were insignificant during cultivation.

Next, we carried out Western blotting on total cellular proteins from clone cl.33-28 of HDAC2(-/-) mutants at shorter intervals up to 56 days, using the chicken IgM L-chain antibody that cross-reacts with IgM H-chain. As shown in Figure 2, protein levels of IgM H-chain and L-chain (detected as two bands) were dramatically increased at the early stage in clone cl.33-28, and thereafter these increased protein levels were gradually reduced via the middle to late stages to almost similar levels in DT40 cells. These results obtained in clones cl.33-12 and cl.33-28 by 2D-PAGE and Western blotting were confirmed in two other independent HDAC2(-/-) mutant clones (cl.33-30 and cl.45-28) (Fig. 3).

Moreover, we examined both subcellular localization and changing pattern of heightened intracellular IgM H-chain in clone cl.33-28 during cultivation by immunocytochemistry with anti-chicken IgM H-chain antibody. Electron microscopy showed that the electron-dense materials were accumulated in irregularly elaborated and vesiculated rough endoplasmic reticula only at the early stage in HDAC2(-/-) mutants (data not shown). Immuno-electron microscopy revealed that colloidal gold immuno-labeling for chicken IgM H-chain was vividly observed in the electron-dense materials accumulating in the dilated and vesiculated rough endoplasmic reticula only at the early (~10 days) stage, and thereafter the heightened protein levels were obviously reduced at the late (~60 days) stage to almost similar levels in DT40 cells (Fig. 4).

These results revealed not only that IgM H- and L-chains are dramatically and considerably accumulated at the early stage of cultivation in HDAC2(-/-) mutant cells, but also that these accumulated immunoglobulin proteins are gradually reduced during cultivation and finally at the late stage reached to comparable levels in DT40 cells. In addition, majority of artificially accumulated IgM H-chain, which existed as a native soluble form to be capable of building a high molecular weight complex with IgM L-chain in HDAC2(-/-) mutants [30], should be localized in rough endoplasmic reticula.

Alterations in gene expressions of IgM H-chain, IgM L-chain and core histones in HDAC2(-/-), respectively, are significant, moderate and insignificant during continuous cultivation

To know alterations in gene expressions of IgM H- and L-chains and core histones in HDAC2(-/-) mutants during cultivation, we prepared total RNAs from three independent clones (cl.33-28, cl.33-30 and cl.45-28) at the early (~20 days), middle (~40 days) and late (~60 days) stages of cultivation and DT40 cells. First, we carried out RT-PCR on total RNAs, using primers IgM Hc, IgM Hs plus IgM Hm and IgM L specific for whole, secreted plus membrane-bound forms of IgM H-chain mRNA and IgM L-chain mRNA (Fig. 5). The levels of whole and secreted forms of IgM H-chain mRNA, respectively,

were obviously and dramatically elevated at the early stage and thereafter gradually reduced via the middle to late stages in these mutant clones, whereas alterations in the levels of membrane-bound form of IgM H-chain mRNA and IgM L-chain mRNA were insignificant and moderate, respectively. These findings in three mutant clones sufficiently agreed with those in clone cl.33-12 (data not shown). Next, we performed RT-PCR on total RNAs, using appropriate primers specific for core histones H2A, H2B, H3 and H4. In three mutant clones, alterations in the mRNA levels of these four core histones were insignificant from the early to late stages (Fig. 5).

Gene expressions of HDAC7, HDAC9 and PCAF in HDAC2(-/-) change during continuous cultivation

To clarify whether alterations in gene expressions of members of chromatin modifying enzymes (HDACs and HATs) are linked with those of IgM H- and L-chains during cultivation, we carried out RT-PCR on total RNAs, using appropriate primers specific for HDAC1, HDAC3, HDAC4, HDAC7, HDAC8, HDAC9 (equivalent to HDAC5 in previous paper [30]), PCAF, GCN5, HAT1, ELP3, TIP60, MORF, MOZ and p300 (Fig. 6A). In three clones cl.33-28, cl.33-30 and cl.45-28, the HDAC9 mRNA level was certainly increased from the early (~20 days) to middle (~40 days) stages of cultivation and thereafter slightly reduced until the late (~60 days) stage. The HDAC7 mRNA level was slightly decreased at the early stage and remained unchanged until the late stage. However, the mRNA levels of residual HDACs did not change. On the other hand, in these mutant clones, the PCAF mRNA level was dramatically increased from the early to late stages of cultivation, whereas those of residual HAT members remained unchanged at any cultivation stages.

Gene expressions of E2A, EBF1, Pax5 and Aiolos in HDAC2(-/-) change differently during continuous cultivation

To clarify whether alterations in gene expressions of various transcription factors are associated with those of IgM H- and L-chains during cultivation, we carried out RT-PCR on total RNAs, using appropriate primers specific for Oct1, Oct2, OBF1, NF-kB, RelB, YY1, NF-AT, PU.1, E2A, EBF1, Pax5 and Aiolos (Fig. 6B). In three clones cl.33-28, cl.33-30 and cl.45-28, the mRNA level of E2A was slightly increased at the early (~20 days) stage of cultivation and thereafter remained unchanged via the middle (~40 days) until late (~60 days) stages. The mRNA level of EBF1 was almost completely decreased at the early stage and thereafter remained unchanged as undetectable level until the late stage. Interestingly, the mRNA level of Pax5 was obviously reduced at the early stage but thereafter certainly elevated via the middle to late stages. The mRNA level of Aiolos was observed to be low at the early stage, and further decreased from the middle to late stages. On the other hand, the mRNA levels of

residual factors (Oct1, Oct2, OBF1, NF-kB, RelB, YY1, NF-AT and PU.1) remained unchanged as a whole during cultivation.

Bulk acetylation levels of some particular Lys residues of core histones in HDAC2(-/-) change during continuous cultivation

To know whether bulk acetylation levels of particular Lys residues (K) of core histones H2A, H2B, H3 and H4 are altered associated with alterations in gene expressions of IgM H- and L-chains during cultivation, we carried out immuno-blotting on total cellular proteins prepared from three clones (cl.33-28, cl.33-30 and cl.45-28) at the early (~20 days), middle (~40 days) and late (~60 days) stages of cultivation, together with those from DT40 cells as control. Site-specific antibodies for various acetylated and methylated Lys residues of core histones H2A, H2B, H3 and H4 were used (Fig. 7). Concerning histone H2A, bulk acetylation levels of K5 and K9 or K7 remained unchanged or slightly changed during cultivation as a whole. Among Lys residues of histone H2B tested, bulk acetylation level of K16 was certainly reduced at the early stage and thereafter remained unchanged until the late stage, whereas those of remaining Lys residues unchanged at any cultivation stages. Bulk acetylation level of K5 of histone H4 was elevated at the early stage and remained unchanged until the late stage, but those of remaining Lys residues of histone H4 unchanged as a whole. Interestingly, concerning histone H3, bulk acetylation levels of K9, K14, K18, K23 and K27 were obviously elevated from the early via middle to late stages, whereas bulk methylation level of K9 did not change. These results obtained here were uncertain yet because some antibodies used were relatively low specificity.

Cloning of ~4.9 kb 5’-upstream region of chicken Pax5 gene

As will be mentioned in our next paper [40], in spite of insufficient information concerning the Pax5 gene located on Z chromosome that is monosomy in chickens, fortunately, we could generate the Pax5-deficient DT40 mutant cell line, Pax5(-), and clarify severe and moderate influences of its deficiency on gene expressions of IgM H- and L-chains and others. In order to know the manner of the Pax5 gene expression, sufficient information on its proximal 5’-upstream region should be essential. However, as mentioned above, nucleotide sequences of the gene were not fully deposited in any database, whereas those on its homologues could be cited from GenBank. Therefore, we directly cloned the ~4.9 kb 5’-upstream region of the Pax5 gene from DT40 genomic DNA by our original gene walking techniques, including Southern blotting, colony hybridization and sub-cloning. Finally, nucleotide sequences of 4950 bp 5’-upstream fragment and 241 bp open reading frame of the chicken Pax5 gene were determined (GenBank accession number: LC060666) (Fig. 8). As a matter of course, nucleotide sequences of XbaI/ClaI fragment agreed fully with those of BamHI/ClaI fragment from positions -1995 to +241. Our nucleotide sequences from positions -635 to +46 were surely coincident with those recently

reported [12]. Dual-luciferase assay using various 3'-deletion and 5'-deletion mutant vectors in both of Hela and DT40 cells preliminarily suggested not only that as a whole the ~4.9 kb 5'-upstream region affected negatively but very slightly on the Pax5 gene expression, but also that two 5'-upstream regions from positions -1561 to -974 and from positions -521 to -40 were possibly involved in negative and bare regulation of the Pax5 gene expression, though these findings are uncertain (our unpublished data).

Acetylation levels of Lys-9 residues of histone H3 within proximal ~2.0 kb 5'-upstream chromatin region of chicken Pax5 gene in HDAC2(-/-) probably change during continuous cultivation

In general, chromatin structure surrounding the proximal 5'-upstream region of a certain gene is thought to participate directly and closely in its gene expression, regardless of the existence or nonexistence of transcriptional element(s) within the region. The above-mentioned unpublished data obtained by the dual-luciferase assay suggested that at least ~1.6 kb 5'-upstream region of the Pax5 gene was necessary for its gene expression, though those were uncertain. Furthermore, remarkably, as shown in Figure 6B, in HDAC2(-/-) mutants the gene expression of Pax5 was decreased at the early cultivation stage and thereafter increased until the late stage. Therefore, to know whether chromatin structure of the Pax5 gene changes during cultivation, we carried out chromatin immuno-precipitation (ChIP) assay, using site-specific antibody for acetylated Lys-9 residue of histone H3 (K9/H3) and appropriate primers (Table II), on its proximal ~2.0 kb 5'-upstream chromatin region in three clones (cl.33-28, cl.33-30 and cl.45-28) at the early (~20 days) and late (~60 days) cultivation stages and DT40 cells as control (Fig. 9). Acetylation levels of K9/H3 within chromatin surrounding the ~2.0 kb 5'-upstream and open reading frame regions (from positions -1923 to +30) of the Pax5 gene were high in DT40 cells. Noticeably, in spite of the HDAC2-deficiency, in these mutant clones, as a whole, acetylation levels of K9/H3 within the ~2.0 kb 5'-upstream chromatin region of the gene were slightly decreased at the early stage and thereafter increased at the late stage, whereas those within the open reading frame remained unchanged. In addition, in clone cl.33-28, acetylation levels of K14/H3 and K18/H3 within the ~2.0 kb 5'-upstream chromatin region of the gene were observed to change slightly at the early and late stages (our unpublished data).

These findings suggested that acetylation levels of at least K9/H3 within the proximal ~2.0 kb 5'-upstream chromatin region of the Pax5 gene in HDAC2(-/-) mutants were reduced at the early cultivation stage and thereafter elevated at the late stage to almost the same levels in DT40 cells. Thus, these results qualitatively agreed with those on alterations in the gene expression level of Pax5 during cultivation.

Discussion

The modulation of chromatin topology with epigenetic modifications, such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation and so on, is one of the most important manners to express cell functions in eukaryotes. For the last several decades, mechanisms to modulate chromatin structure with such epigenetic modifications have been intensively studied without interruptions in diverse life science fields, e.g., gene expression/transcription, DNA replication, differentiation, development, memory, pluri-potency, clinical medicine and so on [1-3, 14-23, 45-72].

We have also systematically studied in vivo roles of numerous members of histones, histone chaperones, HATs, HDACs and transcription factors [24-33, 37-41], using gene targeting techniques in the chicken B cell line DT40 possessing homologous recombination with a very high frequency [34-36]. To know individual roles of transcription factors altered associated with the HDAC2-deficiency on regulation of gene expressions of IgM H- and L-chains, we generated homozygous DT40 mutant cell lines EBF1(-/-), Aiolos(-/-), E2A(-/-) plus Helios(-/-) [30, 31], and also Pax5-deficient mutant cell line Pax5(-) [40] plus Ikaros-down mutant cell line Ikaros(-/-/+) (its generation will be shown elsewhere). Systematic analyses of these resultant mutants showed that Pax5, EBF1, Aiolos plus Ikaros down-regulate gene expressions of IgM H- and L-chains, and E2A up-regulates these two immunoglobulin gene expressions, indicating that HDAC2 regulates indirectly gene expressions of IgM H- and L-chains through opposite regulations of gene expressions of Pax5, EBF1, Aiolos plus Ikaros, and E2A [30, 31].

In this study, through qualitative analyses of HDAC2(-/-) mutant cells collected at different cultivation periods, we casually noticed following surprising phenomena. 2D-PAGE showed not only that amounts of IgM H- and L-chains were dramatically and certainly elevated at the early stage of cultivation, but also that the elevated amounts of these two immunoglobulin proteins were gradually reduced and at the late stage reached to comparable levels in DT40 cells (Fig. 1). On the other hand, cellular levels of most of other major proteins were insignificantly changed during cultivation. Western blotting, using the chicken IgM L-chain antibody that cross-reacts with IgM H-chain (Figs. 2 and 3), sufficiently confirmed the above-mentioned results obtained by 2D-PAGE. Immuno-electron microscopy using antibody specific for chicken IgM H-chain also showed that in HDAC2(-/-) the immunoglobulin proteins were clearly accumulated in endoplasmic reticula at the early stage, and thereafter the accumulated proteins were reduced at the late stage to almost similar level in DT40 cells (Fig. 4). Further, RT-PCR using primers IgM Hc and Hs showed that whole and secreted forms of IgM H-chain mRNA were considerably and dramatically increased at the early stage, and thereafter the increased amounts of these two type mRNAs were gradually decreased via the middle to late stages to very close levels in DT40 cells, although IgM L-chain mRNA was moderately altered during cultivation (Fig. 5). These results meant that gene expressions of IgM H- and L-chains are dramatically and slightly increased at the early stage and thereafter obviously decreased via the middle to late stages to almost

similar levels in DT40 cells.

Interestingly, RT-PCR using appropriate primers specific for various genes of chromatin modifying enzymes (HDACs and HATs) and transcription factors showed noticeable results as follows (Fig. 6). Concerning HATs, in HDAC2(-/-) the gene expression of PCAF was dramatically increased from the early via middle to late stages of cultivation. On the other hand, regarding HDACs, the gene expression of HDAC9 was surely increased from the early to middle stages and thereafter slightly decreased at the late stage, contrary to this, that of HDAC7 was slightly decreased at the early stage and remained unchanged during further cultivation. As for transcription factors, in HDAC2(-/-) the gene expression of EBF1 was almost completely decreased at the early stage and thereafter remained undetectable level until the late stage, but that of E2A was slightly increased at the early stage and remained unchanged during further cultivation. Surprisingly, the gene expression of Pax5 was obviously decreased at the early stage and thereafter certainly increased via the middle to late stages. That is, as a whole, in HDAC2(-/-) the changing pattern of the Pax5 gene expression during cultivation was in anti-parallel with those of IgM H- and L-chain gene expressions. Moreover, immuno-blotting using site-specific antibodies for various acetylated Lys residues (K) of core histones H2A, H2B, H3 and H4 showed that bulk acetylation levels of K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 were gradually elevated during cultivation, although insignificant or slight alterations were observed in those of remaining Lys residues of core histones (except significant changes of K16/H2B and K5/H4) (Fig. 7).

To explore the manner for the gene expression of Pax5, first, we directly cloned and sequenced its ~4.9 kb 5'-upstream fragment from DT40 genomic DNA by our original gene walking techniques including Southern blotting, colony hybridization and sub-cloning (Fig. 8). Dual-luciferase assay showed that the ~4.9 kb 5'-upstream region and especially its two regions from positions -1561 to -974 and from positions -521 to -40 are thought to be possibly necessary for negative control of the Pax5 gene expression (our unpublished data), although these data are still uncertain and critical promoter region(s) or element(s) has not been determined yet. Finally, to know how the gene expression level of Pax5 is altered in HDAC2(-/-) during cultivation, we analyzed chromatin structure surrounding the proximal 5'-upstream region from positions -1923 to +30 of the gene at the early and late stages by the ChIP assay. Interestingly, acetylation levels of K9/H3 within the limited chromatin region were reduced as a whole at the early stage and thereafter elevated at the late stage to almost similar levels in DT40 cells (Fig. 9).

Taken together, these results indicated that the HDAC2-deficiency dramatically and considerably induces accumulations of IgM H- and L-chains based on dramatic and considerable increases in their gene expression (mRNA) levels at the early stage of cultivation, and thereafter these accumulated immunoglobulin mRNAs and proteins are gradually reduced via a lot of generations during continuous cultivation. Such alterations in gene expressions of IgM H- and L-chains should be coupled with those of gene expressions of some of Pax5, Aiolos, EBF1, E2A, PCAF, HDAC7 and HDAC9 and others. In

addition, changes in acetylation levels of K9/H3 within chromatin structure surrounding the proximal ~2.0 kb 5'-upstream region of the Pax5 gene in HDAC2(-/-) should be a basis for changes in its gene expression level through the chromatin structural change, resulting in alterations in gene expressions of IgM H- and L-chains. However, unfortunately, the early cultivation stage in both of RT-PCR and ChIP assay was considerably delayed from that in other four experiments (2D-PAGE, Western blotting, immuno-blotting and immuno-electron microscopy), since the mutant cells used for the former two experiments had been cultivated longer periods than those use for the latter four experiments. Therefore, to further elucidate the above-mentioned interesting biological phenomena, we will newly generate HDAC2-deficient DT40 mutant cells, collect large amounts of them (enough for all experiments) at the early (as early as possible), middle and late cultivation stages, and analyze their cellular characteristics at these three stages in detail.

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Figure legends

Figure 1. Alterations in amounts of IgM H- and L-chain proteins in HDAC2(-/-) DT40 mutants during cultivation

Total cellular proteins were prepared from HDAC2(-/-) mutant clone (cl.33-12) at the early (E; ~10 days), middle (M; ~30 days) and late (L; ~60 days) stages of cultivation and compared with those from DT40 (W) by 2D-PAGE. Iso-electrofocusing in the first dimension (pI) and SDS-PAGE in the second dimension (MW) were performed on Immobiline DryStript gel (pH 4 - 7) and ExcelGel XL SDS gel (gradient 12 - 14), followed by the fluorostaining method. H and L indicate IgM H-chain and L-chain (detected as two spots), respectively.

Figure 2. Alterations in protein levels of IgM H- and L-chains in HDAC2(-/-) DT40 mutants during cultivation

Total cellular proteins were prepared from HDAC2(-/-) mutant clone (cl.33-28) at indicated cultivation periods and compared with those from DT40 (W) by Western blotting. Aliquots of cell extracts were subjected to 12% SDS-PAGE and transferred to membrane filter. Proteins were detected with anti-chicken IgM L-chain antiserum (as primary antibody) that cross-reacts with IgM H-chain. Relative levels of IgM H-chain and large (high) and small (low) forms of IgM L-chain are shown in the lower panel. IgM H and IgM L indicate IgM H- and L-chains, respectively.

Figure 3. Alterations in protein levels of IgM H- and L-chains in two other HDAC2(-/-) DT40 mutant clones during cultivation

Total cellular proteins were prepared from two other HDAC2(-/-) mutant clones (cl.33-30 and cl.45-28) at indicated cultivation periods and analyzed as in Figure 2. However, these periods were not coincident with those in Figure 2.

Figure 4. Alterations in amounts of IgM H-chain protein in HDAC2(-/-) DT40 mutants during cultivation

HDAC2(-/-) mutant clone (cl.33-28) at the early (E; ~10 days) and late (L; ~60 days) stages of cultivation and DT40 (W) were fixed, and immuno-electron microscopy with anti-chicken IgM H-chain antiserum was performed. A) Study in wide range. A number of cells were observed in (W), (E) and (L), but accumulated IgM H-chain proteins were detected only in mutant cells in (E). B) Study for single cell. Each of cells indicated by arrows in A) was magnified and shown in (W), (E) and (L). A large amount of accumulated IgM H-chain proteins were observed only in (E).

Figure 5. Alterations in gene expressions of IgM H- and L-chains, and core histones in

HDAC2(-/-) DT40 mutants during cultivation

Total RNAs were extracted from three HDAC2(-/-) mutant clones (cl.33-28, cl.33-30 and cl.45-28) at the early (E; ~20 days), middle (M; ~40 days) and late (L; ~60 days) stages of cultivation and DT40 (W). RT-PCR was performed on equal amounts of total RNAs, using appropriate primers for HDAC2, whole (IgM Hc), secreted (IgM Hs) plus membrane-bound forms (IgM Hm) of IgM H-chain and IgM L-chain (IgM L), and four core histones (H2A, H2B, H3 and H4). Chicken β-actin was used as control. Some data for DT40 (W) and HDAC2(-/-) at the early (E) stage were identical with those in our previous paper [30].

Figure 6. Alterations in gene expressions of HDACs, HATs and transcription factors in HDAC2(-/-) DT40 mutants during cultivation

Total RNAs were the same ones as in Figure 5. RT-PCR was performed in the same manner using appropriate primers for members of HDACs and HATs (A) and transcription factors (B), instead of primers used in Figure 5. Some data for DT40 (W) and HDAC2(-/-) at the early (E) stage were identical with those in our previous paper [30].

Figure 7. Alterations in bulk acetylation and methylation levels of Lys residues of core histones in HDAC2(-/-) DT40 mutants during cultivation

Whole cellular proteins were extracted from three HDAC2(-/-) mutant clones (cl.33-28, cl.33-30 and cl.45-28) at the early (E; ~20 days), middle (M; ~40 days) and late (L; ~60 days) stages of cultivation and DT40 (W) (three separate cultures) and subjected to 15% SDS-PAGE. Bulk acetylation levels of particular Lys residues (K) (indicated by appropriate designations) of core histones H3, H4, H2A and H2B were measured by immunoblotting using various site-specific anti-acetylated histone antibodies. Bulk methylation level of K9/H3 was measured using site-specific anti-methylated histone antibody. Most data for DT40 (W) and HDAC2(-/-) at the early (E) stage were identical with those in our previous paper [30].

Figure 8. Nucleotide sequences of the 5'-upstream region of chicken Pax5 gene

Nucleotide sequences of the 5'-upstream region of the chicken Pax5 gene up to the position -4950 (including exon 1, putative transcription start site and translation initiation triplet) are shown.

Figure 9. Alterations in acetylation levels of Lys-9 residue of histone H3 within proximal 5'-upstream chromatin region of Pax5 gene in HDAC2(-/-) DT40 mutants during cultivation

Chromatin immunoprecipitation (ChIP) assay was carried out using Chromatin Immunoprecipitation Assay kit. Cross-linked chromatins prepared from cell lysates of three HDAC2(-/-) mutant clones

(cl.33-28, cl.33-30 and cl.45-28) at the early (E; ~20 days) and late (L; ~60 days) stages of cultivation and DT40 (W) were co-precipitated by antiserum specific for acetylated Lys-9 residue of histone H3 (K9/H3). After de-crosslinking, co-precipitated chromatins were amplified by PCR using appropriate primers (see Table II) for segments A to Q of the proximal 5'-upstream region of the Pax5 gene (see Fig. 8). PCR products were analyzed by 1.5% agarose gel electrophoresis.