Chapter 2

Protein and mRNA levels of IgM H- and L-chains artificially accumulated in HDAC2-deficient DT40 mutants are dramatically reduced through various generations during continuous cultivation

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SUMMARY

We analyzed histone deacetylase-2 (HDAC2)-deficient DT40 mutant cells HDAC2(-/-), which were cultivated for varying periods, by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), Western blotting, reverse transcription-polymerase chain reaction (RT-PCR) and immuno-electron microscopy. During continuous cultivation, protein and mRNA levels of IgM H- and L-chains were dramatically and considerably increased at the early stage, and thereafter until the later stage gradually reduced to comparable levels as in DT40 cells. Further, the mRNA level of PCAF was dramatically increased from the early to later stages, and those of HDAC7 and HDAC9 certainly changed. The mRNA level of EBF1 was almost completely decreased at the early stage and thereafter remained unchanged as undetectable level, but that of E2A was slightly increased at the early stage and thereafter remained unchanged. Remarkably, the mRNA level of Pax5, which down-regulates gene expressions of IgM H- and L-chains, was surely reduced at the early stage and thereafter certainly increased through the middle to later stages in anti-parallel with those of these two immunoglobulin proteins. In addition, bulk acetylation levels of K9, K14, K18, K23 and K27 of histone H3 were gradually increased. Subsequently, we cloned ~4.9 kb 5'-upstream region of the Pax5 gene from DT40 genomic DNA. Chromatin immuno-precipitation (ChIP) assay suggested that acetylation levels of Lys-9 residues of histone H3 (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 later stage increased to comparable levels as in DT40 cells.

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

INTRODUCTION

In eukaryotic cells, alterations in the chromatin structure are preferentially involved in controls of gene expressions, and DNA replication, repair and recombination, etc. [1-3]. Naturally, such alterations are concerned in regulations of the lymphocyte development and differentiation [4-7]. Regulations of the lymphocyte development require a number of transcription factors, including Ikaros, E2A, PU.1, GATA-3, Pax5, EBF, etc. [8-13]. Moreover, the transcription regulation of IgM H-chain requires USF, TFEB, Ig/EBP, NF-IL6, OCA-b, etc. as promoter binding proteins and Ig/EBP, NF-IL6, YY-1, E2A, PU.1, etc. as intron enhancer binding proteins. On the other hand, of various epigenetic modifications of the chromatin structure with acetyl, methyl and phosphate groups and others, acetylation and deacetylation of

specific Lys residues (K) of core histones (H2A, H2B, H3 and H4) may be major ones [14-21]. They are cooperatively controlled in combination of several kinds of histone acetyltransferases (HATs) and histone deacetylases (HDACs) [22-25]. To clarify individual roles of members of HATs and HDACs in expressions of normal cell functions, we systematically generated a number of homozygous (or conditional) chicken DT40 mutant cell lines lacking appropriate genes [26-42] by gene targeting techniques [43-53]. Our previous analyses of HDAC2-deficient DT40 mutant cells, HDAC2(-/-), revealed that HDAC2 controls the amount of IgM H-chain at the steps of the transcription of its gene plus the alternative processing of its pre-mRNA [26] and down-regulates IgM L-chain gene promoter activity [29]. 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 specific Lys residues of core histones H2A, H2B, H3 and H4 [33].

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 [33, 54-58]. In addition, we generated Pax5-deficient mutant cells, Pax5(-), devoid of the Pax5 gene existing on Z sex chromosome that is monosomy in chickens (USCS Genome Browser data base) [see Chap. 3], but could generate only Ikaros-down mutant cells, Ikaros(-/-/+), devoid of two alleles of the Ikaros gene existing on chromosome 2 that is trisomy, since it should probably 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 transcriptions of IgM H- and L-chain genes, and E2A up-regulates transcriptions of these two immunoglobulin genes [33]. 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 [33, 36].

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 accumulated at the early stage, and thereafter gradually reduced and finally at the later stage reached comparable levels as in DT40 cells. In addition, mRNA levels of HDAC7, HDAC9 and PCAF were gradually elevated, and those of Pax5, Aiolos, E2A and EBF1 certainly changed 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 later stage increased to comparable levels as in DT40 cells. We discussed on these changing patterns in gene expressions of IgM H- and L-chains, specific 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.

RESULTS

IgM H- and L-chains artificially accumulated in HDAC2(-/-) DT40 mutants are dramatically and 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 later (L; ~60 days) stages of cultivation and from DT40 cells (W) as a control by 2D-PAGE (Fig. 2-1). As reported previously [26], the amounts of IgM H-chain and L-chain (detected as two spots) were dramatically and certainly increased at the early cultivation stage in clone cl.33-12 compared with those as in DT40 cells. Surprisingly, the elevated amounts of these two immunoglobulin proteins in clone cl.33-12 were gradually reduced through the middle to later cultivation stages to almost same levels as in DT40 cells, whereas changes in cellular levels of most of other major proteins were insignificant during cultivation.

Moreover, Western blotting carried out at shorter intervals up to 56 days, using antibody for chicken IgM L-chain that cross-reacts with IgM H-chain, revealed that 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 (Fig. 2-2). Thereafter, these increased protein levels were gradually reduced through the middle to later stages to almost similar levels as in DT40 cells. These results obtained in clones cl.33-12 and cl.33-28 by 2D-PAGE and Western blotting were sufficiently confirmed in two other independent clones cl.33-30 and cl.45-28 (Suppl. Fig. 2-S1).

Next, we examined both subcellular localization and changing pattern of heightened intracellular IgM H-chains in clone cl.33-28 during cultivation by immunocytochemistry analysis 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). Moreover, 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 later (~60 days) stage to almost similar levels as in DT40 cells (Fig. 2-3).

Taken together, 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 later stage reached comparable levels as in DT40 cells. In addition, majority of artificially accumulated IgM

H-chains, which were indicated to exist as a native soluble form to be capable of building a high molecular weight complex with IgM L-chains in HDAC2(-/-) mutants [33], should be localized in specific subcellular organelle, i.e., rough endoplasmic reticula.

Alterations in gene expressions of IgM H-chain, IgM L-chain and core histones in HDAC2(-/-) DT40 mutants, respectively, are significant, moderate and insignificant during continuous cultivation

To know alterations in gene expressions of IgM H- and L-chains and core histones (H2A, H2B, H3 and H4) in HDAC2(-/-) mutants during cultivation, we prepared total RNAs from three independent clones (cl.33-28, cl.33-30 and cl.45-28) of HDAC2(-/-) mutants at the early (~20 days), middle (~40 days) and later (~60 days) stages of cultivation and from 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, respectively, and β-actin was used as a control (Fig. 2-4). 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 through the middle to later 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 using appropriate primers specific for core histones H2A, H2B, H3 and H4 on total RNAs. In three mutant clones, alterations in the mRNA levels of the four core histones were insignificant from the early to later stages (Fig. 2-4).

Gene expressions of HDAC7, HDAC9 and PCAF in HDAC2(-/-) DT40 mutants change during continuous cultivation

To clarify whether alterations in gene expressions 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 our previous paper [33]), PCAF, GCN5, HAT1, ELP3, TIP60, MORF, MOZ and p300, together with β-actin as a control (Fig. 2-5A). 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 later (~60 days) stage. The HDAC7 mRNA level was slightly decreased at the early stage and remained unchanged until the later stage. However, gene expressions 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 later 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(-/-) DT40 mutants differently change 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, together with β-actin as a control (Fig. 2-5B). In three clones cl.33-28, cl.33-30 and cl.45-28, the gene expression of E2A was slightly increased at the early (~20 days) stage of cultivation and remained unchanged through the middle (~40 days) until later (~60 days) stages. The gene expression of EBF1 was almost completely decreased at the early stage and remained unchanged as an undetectable level until the later stage. Interestingly, the gene expression of Pax5 was obviously reduced at the early stage but thereafter certainly elevated through the middle to later stages. The gene expression of Aiolos was observed to be low at the early stage, and further decreased from the middle to later stages. On the other hand, gene expressions 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 specific Lys residues of core histones in HDAC2(-/-) DT40 mutants change during continuous cultivation

To know whether bulk acetylation levels of specific Lys residues (K) of core histones H2A, H2B, H3 and H4 change associated with those of 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 later (~60 days) stages of cultivation, together with those from DT40 cells as a control. Site-specific antibodies for various acetylated Lys residues of core histones H2A, H2B, H3 and H4 were used (Fig. 2-6). Concerning histone H2A, bulk acetylation levels of K5, K7 and K9 remained unchanged (or insignificantly changed) during cultivation as a whole. Among tested Lys residues of histone H2B, the bulk acetylation level of K16 was certainly reduced at the early stage and thereafter remained unchanged until the later stage, whereas those of remaining Lys residues remained unchanged at any cultivation stages. The bulk acetylation level of K5 of histone H4 was elevated at the early stage and remained unchanged until the later stage, but those of remaining Lys residues unchanged as a whole. Interestingly, concerning histone H3, bulk acetylation levels of K9, K14, K18, K23 and K27 were obviously elevated from the early through middle to later stages, whereas the methylation level of K9 did not change. These qualitatively obtained results were uncertain yet, because some of these antibodies were relatively low specificity.

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

As will be mentioned in the next Chapter 3, in spite of insufficient information concerning the Pax5 gene (as a single allele) existing on Z sex 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 the gene expressions of IgM H- and L-chains and others. In order to know the mechanism of the Pax5 gene expression, sufficient information on its proximal 5'-upstream region should be essential. However, at the start of this study, the nucleotide sequences of the Pax5 gene were not fully deposited in any database, whereas those of its homologues could be cited from GenBank. Therefore, we cloned and sequenced the proximal ~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 as follows.

To clone the ~4.9 kb 5'-upstream fragment of the Pax5 gene, we first amplified the 85 bp fragment, corresponding to positions -55 to +30 in exon 1 and including its translation initiation codon, from DT40 genomic DNA by PCR. Since several screenings using the resultant PCR product as a probe yielded no positive clones from a DT40 λFIXII genomic library [26], we directly screened DT40 genomic DNA to obtain the Pax5 5'-upstream fragment in combination with Southern blotting and colony hybridization using the above-mentioned PCR amplified product. As a first step, we digested DT40 genomic DNA in various different combinations of two of numerous restriction enzymes, each of which recognizes multiple cloning sites of pBluescript II SK(+) plasmid, and subsequently performed Southern blotting using the 85 bp PCR amplified fragment as a probe on digested DNA fragments several times. Of numerous positive fragments being different in length and restriction enzyme sites, we focused our attention on the ~2.1 kb XbaI/ClaI fragment containing the 85 bp fragment. To obtain the desired ~2.1 kb fragment, large amounts of genomic DNA (200 μg) were digested with XbaI plus ClaI, and the digested fragments were separated in 0.8% agarose gel, followed by excising the gel piece expected to contain the ~2.1 kb fragments. The DNA fragments were extracted using filter cartridge from the gel piece, recovered by phenol-chloroform extraction plus ethanol precipitation, and inserted into XbaI/ClaI sites of pBluescript II SK(+) vector. The ligated plasmids were introduced into XL-1 blue MRF competent cells, and colony hybridization using the 85 bp fragment as a probe was carried out to yield positive colonies. Then, the recombinant plasmid DNA was isolated and its nucleotide sequences were determined. The XbaI/ClaI fragment consisted of 1995 bp 5'-upstream and 241 bp downstream regions from the transcription initiation site +1.

As a second step, we obtained further extended 5'-upstream fragment of the Pax5 gene, using colony hybridization and sub-cloning techniques as follows. By PCR using appropriate sense and antisense primers constructed based on the above-mentioned nucleotide sequences, the 1626 bp fragment corresponding to positions -1656 to -32 of the gene was amplified from DT40 genomic DNA. The resultant PCR products were digested by BssHII and separated in 0.8% agarose gel. The desired 263 bp

fragment was excised from the gel, purified and sequenced. The obtained BssHII fragment corresponded to the region from positions -1656 to -1394 of the Pax5 gene was used as a probe in subsequent sub-cloning.

To obtain the proximal ~4.9 kb 5'-upstream region of the Pax5 gene, ~4.9 kb BamHI/ClaI fragment hybridized with both the 85 bp fragment and the 263 bp fragment as probes was inserted into pBluescript II SK(+) vector in frame. Screening and cloning techniques were essentially similar to those described above. Finally, we cloned and sequenced the 4950 bp 5'-upstream fragment and the 241 bp open reading frame (ORF) of the chicken Pax5 gene (Suppl. Fig. 2-S2). As a matter of course, the nucleotide sequences of the XbaI/ClaI fragment agreed fully with those of the BamHI/ClaI fragment from positions -1995 to +241. The nucleotide sequences of the ~4.9 kb 5'-upstream region of the chicken Pax5 gene were registered to a database (GenBank accession number: LC060666). Our nucleotide sequences from positions -635 to +46 were surely coincident with those very recently reported [12]. Dual-luciferase assay with several 3'-deletion and 5'-deletion mutant vectors in HeLa and DT40 cells preliminarily suggested not only that as a whole the ~4.9 kb 5'-upstream region negatively but very slightly affected 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 gene expression, though these findings are still 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(-/-) DT40 mutants probably change during continuous cultivation

In general, the chromatin structure surrounding the proximal 5'-upstream region of a certain gene directly and closely participates in its transcription, regardless of the existence or nonexistence of transcriptional element(s) within the region. The above-mentioned unpublished data suggested that at least ~1.6 kb 5'-upstream region of the Pax5 gene is necessary for its transcription, though those are still uncertain. Furthermore, remarkably, as shown in Figure 2-5B, in HDAC2(-/-) mutants the gene expression of Pax5 was decreased at the early cultivation stage and thereafter increased until the later stage. Therefore, to know whether the chromatin structure surrounding the proximal 5'-upstream region of the Pax5 gene changes during cultivation, we carried out chromatin immuno-precipitation (ChIP) assay, using site-specific antibody for the acetylated Lys-9 residue of histone H3 (K9/H3) and appropriate primers (Suppl. Table 2-SII), on the proximal ~2.0 kb 5'-upstream chromatin region of the gene in three clones (cl.33-28, cl.33-30 and cl.45-28) at the early (~20 days) and later (~60 days) cultivation stages and in DT40 cells as a control (Fig. 2-7). Acetylation levels of K9/H3 within the chromatin surrounding the ~2.0 kb 5'-upstream and ORF regions (from positions -1923 to +30) of the Pax5 gene were high in DT40 cells. Surprisingly, in spite of the HDAC2-deficiency, as a whole, acetylation levels of K9/H3 within

the ~2.0 kb 5'-upstream chromatin region of the Pax5 gene in these mutant clones were slightly decreased at the early stage and thereafter increased at the later stage, whereas those within the ORF region 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 slightly change at the early and later 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 later stage to almost the same levels as in DT40 cells. Thus, these results qualitatively agreed with those on alterations in the gene expression level of Pax5.

DISCUSSION

To know individual roles of transcription factors, gene expressions of which changed 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(-/-) [33, 36, 54-58], and Pax5-deficient mutant cell line Pax5(-) [Chap. 3] plus Ikaros-down mutant cell line Ikaros(-/-/+) (its generation will be shown elsewhere). Systematic analyses of these resultant mutants revealed that Pax5, EBF1, Aiolos plus Ikaros down-regulate gene expressions of IgM H- and L-chains, and E2A up-regulates gene expressions of these two immunoglobulin proteins, indicating that HDAC2 indirectly regulates gene expressions of IgM H- and L-chains through opposite regulations of gene expressions of Pax5, EBF1, Aiolos plus Ikaros, and E2A [33, 36].

In this study, throughout qualitative analyses of HDAC2(-/-) mutant cells, which were collected at different cultivation periods, we accidently noticed following surprising phenomena. 2D-PAGE showed not only that amounts of IgM H- and L-chains are dramatically and certainly elevated at the early stage of cultivation, but also that the elevated amounts of these two immunoglobulin proteins are gradually decreased and finally at the later stage reached comparable levels as in DT40 cells (Fig. 2-1). On the other hand, changes in cellular levels of most of other major proteins are insignificant during cultivation. Western blotting carried out at shorter intervals, using antibody for chicken IgM L-chain that cross-reacts with IgM H-chain, sufficiently confirmed the above-mentioned results obtained by 2D-PAGE (Fig. 2-2 and Suppl. Fig. 2-S1). Immuno-electron microscopy using antibody specific for chicken IgM H-chain also showed that in HDAC2(-/-) the immunoglobulin proteins are clearly accumulated in endoplasmic reticula at the early stage, and the accumulated proteins are reduced at the later stage to almost similar level as in DT40 cells (Fig. 2-3). Further, RT-PCR using primers IgM Hc and Hs showed that whole and secreted forms of IgM H-chain mRNA are considerably and dramatically increased at the early stage, and the elevated amounts of these two type mRNAs are gradually reduced through the middle to later stages to very close levels as in DT40 cells (Fig. 2-4).

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. 2-5). Concerning HATs, in HDAC2(-/-) the gene expression of PCAF is dramatically elevated from the early through middle to later stages of cultivation. On the other hand, regarding HDACs, the gene expression of HDAC9 is surely increased from the early to middle stages but thereafter slightly decreased at the later stage, contrary to this, that of HDAC7 is slightly decreased at the early stage and remains unchanged during further cultivation. As for transcription factors, in HDAC2(-/-) the gene expression of EBF1 is almost completely decreased at the early stage and thereafter remains as an undetectable level until the later stage, but that of E2A is slightly elevated at the early stage and remains unchanged during further cultivation. Surprisingly, the gene expression of Pax5 is obviously decreased at the early stage and thereafter certainly increased through the middle to later stages. That is, as a whole, in HDAC2(-/-) the changing pattern of the Pax5 gene expression during cultivation is 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 of core histones H2A, H2B, H3 and H4 showed that bulk acetylation levels of K9, K14, K18, K23 and K27 residues of histone H3 are gradually increased during cultivation, although insignificant changes are observed in those of remaining tested Lys residues (except K16 of histone H2B and probably K5 of histone H4) of core histones (Fig. 2-6).

To explore the way of the chicken Pax5 gene expression, first, we directly cloned and sequenced the proximal ~4.9 kb 5'-upstream fragment of the gene from DT40 genomic DNA by our original gene walking techniques, including Southern blotting, colony hybridization and sub-cloning (Suppl. Fig. 2-S2), since the detailed information on its nucleotide sequences was not deposited in any database at the start of this study. Dual-luciferase assay suggested 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 possibly necessary for negative control of the Pax5 gene expression (our unpublished data), although critical promoter region(s) or element(s) has not been determined yet. Finally, to know how the gene expression level of Pax5 changes in HDAC2(-/-) during cultivation, we analyzed the chromatin structure surrounding the proximal 5'-upstream region from positions -1923 to +30 of the gene at the early and later stages by the ChIP assay. Interestingly, acetylation levels of Lys-9 residues of histone H3 (K9/H3) within the limited chromatin region are decreased at the early stage and thereafter increased at the later stage to almost similar levels as in DT40 cells (Fig. 2-7).

Taken together, these results indicated that the HDAC2-deficiency dramatically and considerably induces accumulations of IgM H- and L-chains depending on dramatic and considerable increases in their gene expressions (mRNAs) at the early stage of cultivation, and thereafter these accumulated immunoglobulin mRNAs and proteins are gradually reduced through various generations during 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, HDAC9 and others. In addition, changes in acetylation levels of at least Lys-9 residues of histone H3 (K9/H3) within the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream region of the Pax5 gene in HDAC2(-/-) should be a basis for changes in the transcription level of the gene through its chromatin structural change, resulting in alterations in gene expressions of IgM H- and L-chains. However, unfortunately, the early cultivation stage in both 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 first two experiments had been already cultivated some times before use of the last four experiments and others. Therefore, to further elucidate the above-mentioned interesting biological phenomena, we must newly generate HDAC2-deficient mutant cells, collect them at early (as quickly as possible), middle and later cultivation stages, and analyze their cellular characteristics in detail at these three stages, each of which is the same cultivation period (as exactly as possible) for all of experiments that will be tested.

METHODS

Cell cultures

Generation of HDAC2(-/-) DT40 mutants was described in our previous paper [26]. All positive mutant clones were 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 [26, 33] and collected at indicated interval periods, including the early (E; ~10 or 20 days), middle (M; ~30 or 40 days) and later (L; ~56 or 60 days) stages of cultivation. The cultivation periods and 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 cells at the early (E; ~10 days), middle (M; ~30 days) and later (L; ~60 days) stages of cultivation and from DT40 cells (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 [26, 46-48, 59].

Western blotting

Cells were cultivated for ~56 days, and whole cellular proteins were prepared from HDAC2(-/-) mutant cells at different cultivation periods and from DT40 cells (W) as described [27, 33]. 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 [30, 33]. Data analyses were carried out by Multi Gauge Ver3.X software using a luminescent image analyzer LAS-1000plus (FUJIFILM) [30, 33]. The relative amounts of IgM H-chain, and large (high) and small (low) forms of IgM L-chain were measured at indicated times.

Immuno-electron microscopy

Post-embedding immunocytochemistry was carried out as described [60]. Exponentially growing DT40 (W) and HDAC2(-/-) mutant cells at the early (E; ~10 days) and later (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 postfixed 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 a 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 later (L; ~60 days) stages of cultivation and from DT40 cells (W) as described [26, 33, 61]. RT-PCR was carried out using appropriate sense and anti-sense primers used in our previous papers [30, 33] and listed in Supplementary Table 2-SI. β-actin was used as a control. 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 [30, 33].

Immunoblotting

Whole cellular proteins were prepared from three independent HDAC2(-/-) clones at the early (E; ~20 days), middle (M; ~40 days) and later (L; ~60 days) stages of cultivation and from DT40 cells (W) (three different cultures) as described [33]. Briefly, cells (1 x 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 specific Lys (K) residues (indicated by appropriate designations) of core histones H3, H4, H2A and H2B, and the bulk methylation level of Lys-9 residue of histone H3 were measured by immunoblotting using site-specific anti-acetylated and anti-methylated histone antibodies as described [33]. 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 proximal ~4.9 kb 5'-upstream fragment of chicken Pax5 gene

Although the chicken Pax5 gene has been reported to exist on Z sex 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 in 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 proximal ~4.9 kb 5'-upstream fragment of the Pax5 gene, we first screened the DT40 genomic DNA library in a λFIXII 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 [62], sub-cloning and colony hybridization (details will be shown later). First, the DT40 genomic DNA was digested with appropriate restriction enzymes, recognizing specific positions in the multiple cloning site of pBluescript II SK(+) vector, and hybridized with the PCR amplicon (as a probe), corresponding to the 85 bp fragment of exon 1 (from positions -55 to +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 bellow. Enough amounts of the

genomic DNA (200 μg) were digested with XbaI and ClaI at 37 ℃ for 18 hrs and separated in a 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 hrs. The ligated plasmid was introduced into XL-1 blue MRF Competent cells, 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 a 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 second step, using the above-mentioned techniques, we cloned and identified the 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 to -32) was obtained from DT40 genomic DNA by PCR using these primers and digested with BssHII. After separation in 0.8% agarose gel, the band of the desired 263 bp fragment was excised from the gel and purified using Wizard SV Gel and PCR Clean-Up System (Promega).

The resultant 263 bp DNA fragment, corresponding to the region from positions -1656 to -1394, was used as a probe for subsequent hybridization to obtain more additional 5'-upstream genomic fragment cut with appropriate restriction enzymes. Among the 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), introduced into XL-1 blue MRF Competent cells, 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 this 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 plates for 45 sec until it became thoroughly wetted and 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 denaturizing 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 later (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 aprotinin (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 the chromatin DNA into lengths 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. The 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 hrs 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 an inner carrier. Anti-AcK9H3 (#07-352) (Millipore) was used for pulldown. Recovered DNA fragments were subjected to PCR for 25 ~ 35 cycles, using several appropriate primers specific for approximately 150 ~ 250 bp segments, corresponding to the proximal 5'-upstream region of the Pax5 gene (from the start codon) and first exon, which are listed in Supplementary Table 2-SII. PCR was carried out using the same master reaction mixture in a 25-μl scale. The immuno-precipitated chromatin treated with non-immune rabbit serum (Vector Laboratories) (as a negative control) produced no bands following PCR (data not shown).

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FIGURE LEGENDS

Figure 2-1. Alterations in amounts of IgM H- and L-chains in HDAC2(-/-) DT40 mutant cells during continuous cultivation

Total cellular proteins were prepared from HDAC2(-/-) mutant cells (clone cl.33-12) at the early (E; ~10 days), middle (M; ~30 days) and later (L; ~60 days) stages of cultivation and compared with those from DT40 cells (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- and L-chains, respectively.

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

Total cellular proteins were prepared from HDAC2(-/-) mutant cells (clone cl.33-28) collected at indicated cultivation periods and compared with those from DT40 cells (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 2-3. Alterations in amounts of IgM H-chain proteins in HDAC2(-/-) DT40 mutant cells during continuous cultivation

HDAC2(-/-) mutant cells (clone cl.33-28) collected at the early (E; ~10 days) and later (L; ~60 days) stages of cultivation and from DT40 cells (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) or (L). A large amount of accumulated IgM H-chain proteins were observed only in (E).

Figure 2-4. Alterations in gene expressions of IgM H- and L-chains, and core histones in HDAC2(-/-) DT40 mutant cells during continuous 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 later (L; ~60 days) stages of cultivation and from DT40 cells (W). RT-PCR was performed using equal amounts of total RNAs and appropriate primers for HDAC2, whole (IgM Hc), secreted (IgM Hs) plus membrane-bound (IgM Hm) forms 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 a

control. Some data for DT40 (W) and HDAC2(-/-) at the early (E) stage were the same as those in our previous paper [33].

Figure 2-5. Alterations in gene expressions of various members of HDACs, HATs, and transcription factors in HDAC2(-/-) DT40 mutant cells during continuous cultivation

Total RNAs used were the same ones as in Fig. 2-4. RT-PCR was performed in the same way, using appropriate primers for various members of HDACs and HATs (A) and transcription factors (B), instead of primers used in Fig. 2-4. Some data for DT40 (W) and HDAC2(-/-) at the early (E) stage were the same as those in our previous paper [33].

Figure 2-6. Alterations in bulk acetylation and methylation levels of various Lys residues of histone H3 in HDAC2(-/-) DT40 mutant cells during continuous 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 later (L; ~60 days) stages of cultivation and from DT40 cells (W) (three separate cultures), and subjected to 15% SDS-PAGE. Bulk acetylation levels of specific Lys (K) residues (indicated by appropriate designations) of core histones H3, H4, H2A and H2B were measured by immunoblotting using various site-specific anti-acetylated histone antibodies. The 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 the same as those in our previous paper [33].

Figure 2-7. Alterations in acetylation levels of Lys-9 residues of histone H3 within proximal 5'-upstream chromatin region of Pax5 gene in HDAC2(-/-) DT40 mutant cells during continuous 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 later (L; ~60 days) stages of cultivation and from DT40 cells (W) were co-precipitated by antiserum specific for acetylated Lys-9 residue of histone H3 (K9/H3). After decross-linking, co-precipitated chromatins were amplified by PCR using appropriate primers A to Q (Suppl. Table 2-SII) for the proximal 5'-upstream region of the Pax5 gene (see Suppl. Fig. 2-S2). PCR products were analyzed by 1.5% agarose gel electrophoresis.

Supplementary Figure 2-S1. Alterations in protein levels of IgM H- and L-chains in two other HDAC2(-/-) DT40 mutant clones during continuous cultivation

Total cellular proteins were prepared from HDAC2(-/-) mutant clones (cl.33-30 and cl.45-28)

collected at indicated cultivation periods and analyzed in the same way in Fig. 2-2. However, these periods were not coincident with those in Fig. 2-2.

Supplementary Figure 2-S2. Nucleotide sequences of the proximal 5'-upstream region of chicken Pax5 gene

Nucleotide sequences of the proximal 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.