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IgM H- and L-chains artificially and excessively accumulated in HDAC2(-/-) DT40 mutants are gradually and dramatically reduced in distinct manners in individual mutant clones via a lot of generations during continuous cultivation**

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****The article is the modified version of Chapter 4 of the self-publishing monograph as follows: Nakayama, M. and Nakayama, T.: Artificially and excessively accumulated IgM H- and L-chains in HDAC2(-/-) DT40 mutants are gradually and dramatically reduced in distinct manners in individual mutant clones via a lot of generations during continuous cultivation. In: Chromatin Conformation Change Code (4C) Theory on Gain of New Cell Function through Irreversible Creation of Chromatin Structure Plasticity with Epigenetic Modifications via a Lot of Generations, Nakayama, T. and Nakayama, M. (Eds.), Miyakonojoh-Insatsu, Inc. Miyazaki, Japan, pp. 71-103, 2015. Available from two URLs (http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10123995 and/or http://hdl.handle.net/10458/5293).**

Abstract

We newly generated HDAC2-deficient DT40 mutants HDAC2(-/-) and analyzed their characteristics at various intervals during cultivation. In six independent clones (cl.2-1 to cl.2-6) of HDAC2(-/-) mutants, proteins and mRNAs of IgM H- and L-chains were dramatically increased at the early stage of cultivation, and thereafter decreased in almost similar changing pattern and at the late stage reached to comparable levels in DT40 cells. By contrast, mRNAs of various transcription factors and chromatin modifying enzymes showed distinct changing patterns in these six HDAC2(-/-) clones during cultivation. In clone cl.2-1, mRNAs of Pax5, Aiolos and EBF1 were almost completely decreased at the early stage and remained unchanged during cultivation, but that of OBF1 was dramatically decreased until the late stage. In clones cl.2-2, cl.2-3, cl.2-4 and cl.2-5, mRNAs of Pax5, Aiolos and EBF1 were almost completely decreased at the early stage, and thereafter mRNAs of Pax5 and Aiolos were increased until the late stage but that of EBF1 remained unchanged during cultivation. In clone cl.2-6, mRNAs of Pax5, Aiolos and EBF1 were almost completely decreased at the early stage and thereafter dramatically increased during cultivation. These findings suggested that three distinct manners for gene expressions of IgM H- and L-chains exist at the late stage in six individual HDAC2(-/-) clones; i.e., clone cl.2-1 seems OBF1-dependent type, clones cl.2-2, cl.2-3, cl.2-4 and cl.2-5 seem Pax5- and Aiolos-dependent type, and clone cl.2-6 seems Pax5-, Aiolos- and EBF1-dependent type. Based on these results, we propose a hypothesis that individual clones of HDAC2(-/-) mutants possess the ability for gain of new cell function to exclude excessively accumulated IgM H- and L-chains as uncomfortable environment change based on their decreased gene expressions through distinct manners to alter gene expressions of these particular transcription factors via a lot of generations.

Keywords

Gene targeting techniques; HDAC2-deficient DT40 mutants; Continuous cultivation; Decreases in IgM H/L chain protein/mRNA levels; Changes in mRNA levels of transcription factors in individual mutant clones

Introduction

Approximately 50 years ago, chemical modifications of histones with acetyl and methyl groups were first proposed to be of fundamental importance as to the regulation of RNA synthesis in eukaryotes [1]. Since then, the modulation of chromatin topology has been undoubtedly thought to be one of the most fundamental and important manners for expression of cell functions in eukaryotes. For the last several decades, mechanisms to modulate chromatin structure with epigenetic modifications, such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation and so on, have been intensively studied in a variety of research fields of life science. Of these epigenetic modifications, acetylation and

deacetylation of particular Lys residues of core histones catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs) as chromatin modifying enzymes are surely major ones [2-14]. Even in the last few years, countless numbers of researches on such epigenetic modifications of chromatin have been vigorously continued without interruptions in more diverse life science fields, e.g., gene expression/transcription, DNA replication, differentiation, development, memory, pluripotency, clinical medicine and so on [15-36].

Using gene targeting techniques in the chicken B cell line DT40 possessing homologous recombination with a very high frequency [37-39], we have also systematically studied in vivo roles of a large number of particular members of HATs, HDACs and transcription factors [40-48], besides histones and histone chaperones [49-56]. Our analyses of initially generated HDAC2-deficient DT40 mutants, HDAC2(-/-), showed that HDAC2 controls the amount of IgM H-chain at the steps of its gene expression and pre-mRNA alternative processing [40]. Moreover, we revealed that the HDAC2-deficiency increases gene expressions of HDAC4, HDAC9, PCAF plus E2A as well as IgM H- and L-chains, decreases those of HDAC7, Pax5, Aiolos, Ikaros plus EBF1, and changes bulk acetylation levels of several particular Lys residues (K) of core histones H2A, H2B, H3 and H4 [41]. Therefore, to know respective roles of these altered transcription factors and others in regulations of gene expressions of IgM H- and L-chains, we successively generated homozygous DT40 mutant cell lines EBF1(-/-), Aiolos(-/-), E2A(-/-) and Helios(-/-), devoid of EBF1, Aiolos, E2A and Helios genes, respectively [41, 43-46]. In addition, we also generated Pax5-deficient mutant cell line Pax5(-), devoid of the Pax5 gene [47] located on Z chromosome that is monosomy in chickens (USCS Genome Browser data base) and Ikaros-down mutant cell line Ikaros(-/-/+), devoid of two alleles of the Ikaros gene (our unpublished data) located on chromosome 2 that is trisomy. Analyses of these resultant mutants revealed that Pax5, EBF1, Aiolos and Ikaros down-regulate gene expressions of IgM H- and L-chains, and E2A up-regulates gene expressions of these two immunoglobulin proteins [41]. Thus, these results obtained from the above-mentioned DT40 mutant cell lines each devoid of HDAC2 and several transcription factors revealed 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 [40, 41, 47, 48].

Surprisingly, during our latest studies on characteristics of the initially generated HDAC2(-/-) DT40 mutants [40, 41], which were cultivated for different periods, we casually noticed following remarkable phenomena [42]. 2D-PAGE showed that in HDAC2(-/-) mutants IgM H- and L-chains were dramatically elevated at the early stage of cultivation and thereafter gradually reduced during cultivation and at the late stage reached to comparable levels in DT40 cells, whereas changes in cellular levels of most of other major proteins were insignificant during cultivation. Western blotting carried out at shorter intervals, using antibody specific for chicken IgM L-chain that cross-reacts with IgM H-chain, sufficiently confirmed the above-mentioned results obtained by 2D-PAGE. Immuno-electron

microscopy using antibody specific for chicken IgM H-chain also showed that the immunoglobulin proteins were clearly accumulated at the early stage and thereafter reduced at the late stage to almost the same level in DT40 cells. RT-PCR using primers IgM Hc and IgM Hs showed that whole and secreted forms of IgM H-chain mRNA were dramatically increased at the early stage, and thereafter gradually decreased during cultivation and at the late stage reached to very close levels in DT40 cells. These results, together, indicated not only that in HDAC2(-/-) mutants IgM H- and L-chains were dramatically and considerably accumulated based on their increased gene expressions at the early stage of cultivation, but also that these accumulated proteins were gradually reduced based on their decreased gene expressions during cultivation and at the late stage finally reached to comparable levels in DT40 cells. Remarkably, RT-PCR, using appropriate primers specific for various genes encoding particular members of HDACs, HATs and transcription factors, showed that gene expressions of PCAF, HDAC7, HDAC9, EBF1, Pax5, E2A, Aiolos and others were separately altered in different manners in HDAC2(-/-) mutants during cultivation. Immuno-blotting, using site-specific antibodies for various acetylated Lys residues (K) of histones H2A, H2B, H3 and H4, showed that bulk acetylation levels of K9, K14, K18, K23 and K27 residues of histone H3 were gradually increased during cultivation, whereas insignificant changes were observed for most of examined Lys residues of other core histones. Furthermore, we cloned and sequenced ~4.9-kb 5'-upstream region of the Pax5 gene from DT40 genomic DNA by our original gene walking techniques (GenBank accession no.: LC060666). Interestingly, qualitative chromatin immuno-precipitation (ChIP) assay suggested that acetylation levels of Lys-9 residue of histone H3 (K9/H3) within some segments of chromatin structure surrounding proximal ~2.0-kb 5'-upstream region of the Pax5 gene were decreased at the early stage in HDAC2(-/-) mutants and at the late stage increased to comparable levels in DT40 cells. Of these newly obtained results from the initially generated HDAC2(-/-) mutants, it should be worth of special mention that artificially and excessively accumulated IgM H- and L-chains at the early cultivation stage were diminished based on their decreased gene expressions, mainly attributed to altered gene expressions of the above-mentioned particular transcription factors during cultivation [42]. Besides, in Pax5(-) mutants these two immunoglobulin proteins artificially and dramatically accumulated at the early stage were more rapidly reduced based on their decreased gene expressions, probably attributed to altered gene expressions of some of particular transcription factors and chromatin modifying enzymes during cultivation [47].

In this study, to clarify manners for decreases in gene expressions of IgM H- and L-chains during cultivation in further detail, we achieved a second generation of homozygous HDAC2-deficient DT40 mutants HDAC2(-/-) by gene targeting techniques [37-39], because the techniques were very powerful for studying physiological roles of numerous genes of HATs, HDACs, transcription factors, histones and histone chaperones as described above [40-56]. Remarkably, protein and mRNA levels of IgM H- and L-chains were dramatically and considerably increased at the early stage of cultivation, and thereafter

gradually and drastically decreased until the late stage in almost similar changing pattern in all of six tested independent clones of HDAC2(-/-) mutants. By contrast, surprisingly, these individual HDAC2(-/-) clones each showed distinct changing patterns in gene expressions of Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, OBF1, Oct2, Blimp1, PCAF, HDAC7 and HDAC9 during cultivation. These results, together with our previous findings [40-42, 47], revealed that in individual clones of HDAC2(-/-) mutants IgM H- and L-chains artificially accumulated at the early cultivation stage were diminished in almost the same changing pattern based on their decreased gene expressions, which were mainly attributed to varied alterations in respective gene expressions of Pax5, Aiolos, EBF1, OBF1 and others among those of the above-mentioned altered transcription factors and chromatin modifying enzymes during cultivation. Based on these results, we propose a hypothesis that individual clones of HDAC2(-/-) mutants acquire the ability to gain new cell function to exclude artificially accumulated IgM H- and L-chains through distinct manners to alter gene expressions of Pax5, Aiolos, EBF1, OBF1 and others probably based on their chromatin conformation changes via a lot of generations during continuous cultivation.

Methods

Cell cultures

HDAC2(-/-) DT40 mutant cells newly generated as shown below were continuously cultivated as described [40-42, 47] and collected at indicated interval periods, including the early (E), middle (M) and late (L) stages of cultivation. These cultivation stages and/or periods were practically counted from the first day of cultivation from the stock at -80°C. DT40 (W) was used as control. Cell numbers were counted at indicated times nearby the late stage to determine growth rate as described [40, 41, 47].

Generation of HDAC2-deficient DT40 mutant cells HDAC2(-/-)

To construct HDAC2-disruption vectors ΔHDAC2MerCreMer/bleo and ΔHDAC2/hyg, the 3.5-kb 5'-upstream (as 5'-arm) and 2.3-kb 3'-downstream (as 3'-arm) fragments were excised from the genomic DNA clone containing the HDAC2 gene previously cloned by us [40] using appropriate restriction enzymes (SpeI plus SphI, and SphI plus BamHI) and transferred into pBluescript II vector as described [40, 41]. The hygromycin (hyg) cassette flanked by a loxP site or MerCreMer/bleo cassette (a kind gift from Dr. M. Reth; Nucleic Acids Res. 1996) was inserted between the 5'-upstream and 3'-downstream fragments. In the resultant targeting vectors, therefore, genomic nucleotide sequences corresponding to exons 6 and 7 of the gene were replaced with the drug resistance cassettes, since the 3.5-kb and 2.3-kb fragments fully corresponded to parts of introns 2 to 5, and intron 7, respectively (Fig. 1A). Transfection was carried out as described [37-41, 49]. To obtain HDAC2-deficient DT40 mutants, transfectants with ΔHDAC2MerCreMer/bleo vector were first selected in medium containing 400 μg of Zeocine per ml. Successively, we transfected the ΔHDAC2/hyg vector into one of the transfectants, in

which one of two HDAC2 alleles had already been disrupted, and then selected stable transfectants in medium containing 400 μg of Zeocine and 2 mg of hyg per ml, respectively. At these targeting steps, we confirmed the disruption of first and second alleles of the HDAC2 gene by Southern blotting using probe HDAC2 as described below. All positive mutant clones were picked up on 96-well plates at ~8 days after knockout of two HDAC2 alleles, cultivated for another ~4 days to be ~10^6 cells/ml and stocked at -80°C until use.

Southern blotting

Southern blotting was performed as described [40, 41, 57]. Genomic DNAs were isolated from DT40 cells and HDAC2(-/-) mutant clones, digested with indicated enzymes (BamH1 and EcoRV), separated in 0.8% agarose gels, electro-transferred to Hybond N+ membranes and hybridized with ^{32}P-labeled probe HDAC2. Probe HDAC2 was the 0.4-kb BamHI/SpeI fragment, corresponding to the 5'-outer side of 5'-arm (and to 3'-downstream of exon 2) within intron 2 of the HDAC2 gene.

RT-PCR

Total RNAs were isolated from six independent HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) at indicated periods, including the early (E; 7 days), middle (M; 33 days) and late (L; 64 days) cultivation stages and DT40 cells (W). RT-PCR was carried out using appropriate sense and anti-sense primers listed in Table I and also our previous papers as described [41, 42, 47, 58]. HDAC2 and β-actin were used as negative and positive controls, respectively. RT-PCR products were subjected to 15% agarose gel electrophoresis. Nucleotide sequences of all amplified products were confirmed by the PCR sequencing protocol as described [41, 58]. Data analyses were carried out by Multi Gauge Ver3.X software using a luminescent image analyzer LAS-1000plus (FUJIFILM). Data calibrated with the control for mutant clones were qualitatively indicated as percentages of control values obtained from DT40 cells.

Western blotting

Western blotting was performed as described [40-42, 47]. Whole cellular proteins were prepared from six HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) at indicated periods, including the early (E; 3 days), middle (M; 29 days) and late (L; 53 days) cultivation stages, and DT40 cells (W) (5 x 10^6 cells), followed by lysis in 50 μl of SDS buffer. Aliquots (10 μl) of 1:25 dilution of the resultant cell extracts were subjected to 12% SDS-PAGE and electro-transferred to membranes. The proteins were successively detected on the same membranes, using rabbit anti-chicken IgM L-chain antiserum that cross-reacts with the IgM H-chain, anti-chicken IgM H-chain antiserum (as primary antibodies) and finally antiserum for β-actin as control. Antibodies used were: A30-100A for IgM L-

and H-chains, A30-102A for IgM H-chain (Bethyl Laboratories Inc., TX, USA) and ab6276 for β-actin (Abcam).

Microscopy, electron microscopy and immuno-electron microscopy

Microscopy, electron microscopy and immuno-electron microscopy using rabbit anti-chicken IgM H-chain antiserum were carried out essentially as described [42, 43, 47, 59, 60]. Briefly, post-embedding immunocytochemistry using immunogold labeling was carried out as follows. HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) at the early (E; ~5 days) and late (L; ~60 days) cultivation stages and DT40 cells (W), all of which were exponentially growing, were collected by a light centrifugation. 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 graded 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.

Results

Genomic organization of chicken HDAC2 gene and generation of homozygous HDAC2-deficient DT40 mutant cells HDAC2(-/-)

As shown in Figure 1A, genomic DNA of the chicken HDAC2 consists of 16 exons, different from 14 exons reported in our previous paper [40]. We newly generated homozygous HDAC2-deficient DT40 mutant cells HDAC2(-/-) devoid of two HDAC2 alleles by gene targeting techniques as described [37-41, 49-56]. To eliminate effects of drug resistant genes in targeting vectors, we first constructed two targeting vectors containing MerCreMer/bleo and hyg, which were different from those used previously [40]. Then, we sequentially introduced these two targeting vectors into DT40 cells derived from

chicken B lymphocyte cells [37-41]. By Southern blotting using probe HDAC2, we isolated 28 independent drug-resistant clones, which lost exons 6 and 7 of two alleles of the HDAC2 gene (data not shown). Among these independent mutant clones, typical patterns of Southern blotting of six clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) are shown in Figure 1B. In all of these six clones, endogenous 4.0-kb band derived from intact alleles disappeared and 7.0-kb band derived from two targeted alleles newly appeared. Residual 22 clones also showed the same results (data not shown). In addition, as expected, RT-PCR using primers specific for HDAC2 showed no band for mRNA originated from the intact HDAC2 gene in the six clones (Fig. 1D), confirming that these six clones (and residual 22 clones) are homozygous HDAC2-deficient DT40 mutant cells HDAC2(-/-). Hereafter, we analyzed several characteristics of these six HDAC2(-/-) mutant clones in detail.

Protein and mRNA levels of IgM H- and L-chains in HDAC2(-/-) mutants change during continuous cultivation

First, to explore whether protein levels of IgM H- and L-chains change in HDAC2(-/-) mutants during cultivation, we carried out Western blotting, using two antibodies specific for chicken IgM L-chain and IgM H-chain, on total cellular proteins prepared from six HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) collected at the early (E; 3 days), middle (M; 29 days) and late (L; 53 days) stages of cultivation, respectively, regarding as cultivation periods of ~5 days, ~30 days and ~50 to 60 days, and DT40 cells (W; wild-type) as control (Fig. 1C). These three cultivation stages were practically counted from the first day of cultivation from the stock at -80°C. As control, β-actin was finally detected with its specific antibody on the same membrane filters. The protein level of IgM H-chain at the early (E) stage in the six HDAC2(-/-) mutant clones was dramatically increased compared with that in DT40 cells (W), and thereafter the increased protein level was gradually decreased in all of the tested mutant clones during cultivation. That is, the IgM H-chain level was moderately decreased at the middle (M) stage and dramatically decreased at the late (L) stage to comparable level in DT40 cells. The protein level of IgM L-chain (detected as two bands) at the early (E) stage in these HDAC2(-/-) mutant clones was considerably increased compared with that in DT40 cells, and the increased protein level was gradually decreased in all of these mutant clones during cultivation. That is, the IgM L-chain level was moderately decreased at the middle (M) stage and considerably decreased at the late (L) stage to very close level in DT40 cells.

Next, to know whether mRNA levels of IgM H- and L-chains change in HDAC2(-/-) mutants during cultivation, we carried out RT-PCR using appropriate primers specific for chicken IgM H- and L-chains on total RNAs prepared from the six HDAC2(-/-) mutant clones, all of which were cultivated for 7 days as the early (E) stage, 33 days as the middle (M) stage and 64 days as the late (L) stage and DT40 cells (W) as control (Fig. 1D). As described previously [40-42, 47], primers IgM Hc, IgM Hs plus IgM Hm

and IgM L could detect specifically whole, secreted plus membrane-bound forms of IgM H-chain mRNA and IgM L-chain mRNA, respectively. As control, β-actin was used, since its mRNA level at the early (E), middle (M) and late (L) stages in the six mutant clones was usually kept constant in the range of approximately 85 ~ 110% of wild-type value in DT40 cells. The levels of whole and secreted forms of IgM H-chain mRNA were drastically increased at the early (E) stage in all of the six mutant clones compared with those in DT40 cells. Thereafter, the increased levels of these two type mRNAs in all of the mutant clones were considerably decreased at the middle (M) stage and dramatically decreased at the late (L) stage to very close levels in DT40 cells. On the other hand, as a whole the level of membrane-bound form of IgM H-chain mRNA insignificantly changed in five mutant clones (cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) during cultivation but fairly decreased via the middle (M) to late (L) stages to be about 50% in mutant clone cl.2-1. In addition, in all of the six mutant clones the level of IgM L-chain mRNA changed slightly or remained unchanged at any cultivation stages.

Increased protein levels of IgM H- and L-chains caused by HDAC2-deficiency are dramatically and gradually decreased in individual clones of HDAC2(-/-) mutants during continuous cultivation

The above-mentioned results that protein and mRNA levels of IgM H- and L-chains in HDAC2(-/-) mutants dramatically changed during cultivation must be very interesting, surprising and important phenomena. To confirm these findings, we carried out Western blotting more minutely at shorter intervals, using two antibodies specific for chicken IgM H-chain and considerably specific for chicken IgM L-chain that cross-reacts with IgM H-chain. Total cellular proteins were prepared from six HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) cultivated for various indicated periods, including the early (E; 3 days), middle (M; 29 days) and late (L; 53 days) stages and DT40 cells (W) (Fig. 2). As control, β-actin was finally detected using its specific antibody on the same membrane filters. As expected, analyses using antibody for chicken IgM L-chain showed not only that the protein level of IgM H-chain (indicated by lower bands) was dramatically increased at the early (E) stage in all of the six mutant clones, but also that the increased protein level was drastically decreased until the late (L) stage to almost the same level in DT40 cells via the in-between cultivation periods including the middle (M) stage. Similarly, analyses using antibody specific for chicken IgM H-chain showed that the protein level of IgM H-chain (indicated by upper bands) was dramatically increased at the early (E) stage, and thereafter the increased protein level was gradually decreased to almost the same level in DT40 cells by 18 ~ 24 days. In addition, as expected, the antibody specific for IgM L-chain showed that the protein level of IgM L-chain (detected as two bands) was certainly increased at the early (E) stage in the six mutant clones, and thereafter the increased protein level was gradually decreased via the in-between cultivation periods including the middle (M) stage until the late (L) stage to almost the same level in DT40 cells. These results, together, revealed that artificially increased protein levels of IgM H- and

L-chains at the early (E) stage in all of the tested six clones of HDAC2(-/-) mutants were surely and dramatically decreased in almost similar changing pattern during cultivation.

Gene expressions of various chromatin modifying enzymes (HDACs and HATs) and transcription factors change in individual clones of HDAC2(-/-) mutants during continuous cultivation

To know whether mRNA levels of chromatin modifying enzymes change in HDAC2(-/-) mutants during cultivation, we carried out RT-PCR, using appropriate primers specific for various genes encoding particular members of chicken HDACs and HATs, on total RNAs prepared from six HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6), which were cultivated for 7, 33 and 64 days as the early (E), middle (M) and late (L) stages, respectively, and DT40 cells (W) (Fig. 3A). In the six mutant clones, mRNA levels of HDAC7, HDAC9 and PCAF changed significantly during cultivation, but those of residual HDACs (HDAC1, HDAC3, HDAC4 and HDAC8) and HATs (GCN5, HAT1, ELP3, MORF, MOZ, TIP60 and p300) remained unchanged or very slightly changed. Concerning the changing patterns in HDAC7, HDAC9 and PCAF gene expressions, these six mutant clones could be roughly classified into three different types; i.e., clone cl.2-1, clones cl.2-2, cl.2-3, cl.2-4 plus cl.2-5, and clone cl.2-6. Detailed changing patterns in their gene expressions in four mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) will be shown later.

To know whether mRNA levels of transcription factors change in HDAC2(-/-) mutants during cultivation, we carried out RT-PCR on the above-mentioned total RNAs, using appropriate primers specific for various transcription factor genes (Fig. 3B). In one or more of the six mutant clones, mRNA levels of Pax5, Aiolos, EBF1, E2A, PU.1, Blimp1 and OBF1 changed during cultivation, but those of residual factors (Oct1, Oct2, NF-kB, RelB, NF-AT, YY1, XBP-1, Stat5 and CstF-64) remained unchanged. Remarkably, concerning the changing patterns in gene expressions of these altered factors, the six mutant clones could be also classified into three different types; i.e., clone cl.2-1, clones cl.2-2, cl.2-3, cl.2-4 plus cl.2-5, and clone cl.2-6, agreed with the above-mentioned classification based on the changing patterns for HDACs and HATs. Because four mutant clones cl.2-2, cl.2-3, cl.2-4 and cl.2-5 had great resemblance in many cellular properties with each other as mentioned above, hereafter, the changing patterns in gene expressions of the altered particular transcription factors and some other cellular characteristics of four mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) will be shown in detail.

Morphology of HDAC2(-/-) mutant cells changes during continuous cultivation

Immuno-electron microscopy, using antibody specific for chicken IgM H-chain, showed that in initially generated HDAC2(-/-) mutant cells [40] the immunoglobulin proteins were clearly accumulated at the early (E) stage and thereafter obviously reduced at the late (L) stage to almost the same level in DT40 cells (W) [42]. To confirm these findings and to explore whether newly generated HDAC2(-/-)

mutant cells morphologically change during cultivation, we first examined morphology of four mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) and DT40 cells as control. Electron microscopy showed that all of these mutant clones were observed to be somewhat distorted form at the early (E) stage but at the late (L) stage to be smooth form, like that of DT40 cells (Fig. 4; upper panels). In addition, dense cytoplasmic fractions, probably due to artificially accumulated IgM H- and L-chains, were observed only at the early (E) stage but not at the late (L) stage as well as in DT40 cells. Immuno-electron microscopy using antibody specific for chicken IgM H-chain showed that IgM H-chain proteins were certainly accumulated at the early (E) stage in all of these mutant clones, and thereafter most of the accumulated proteins disappeared at the late (L) stage, like in DT40 cells (Fig. 4; lower panels). These results roughly but surely agreed with those in initially generated HDAC2(-/-) mutants mentioned above [42]. Next, we carried out microscopy on four HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) at the early (E) and late (L) cultivation stages and DT40 cells (Fig. 5). Interestingly, these mutant clones were morphologically observed to be aggregative form at the early (E) stage but dispersive form at the late (L) stage, like the form of DT40 cells and also that of Pax5(-) mutant cells [47].

Gene expressions of IgM H- and L-chains change in almost similar pattern in individual clones of HDAC2(-/-) mutants during continuous cultivation

As shown in Figures 3A and 3B, mRNA levels of PCAF, HDAC7, HDAC9, Pax5, Aiolos, EBF1, E2A, PU.1, Blimp1 and OBF1, as well as protein and mRNA levels of IgM H- and L-chains (Figs. 1C, 1D and 2), were altered in the six tested HDAC2(-/-) mutant clones during cultivation. We examined more minutely changing patterns in gene expressions of IgM H- and L-chains and those of altered members of HATs, HDACs and transcription factors (and also Ikaros and XBP-1) in four HDAC2(-/-) mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 during cultivation. Total RNAs were prepared from these four mutant clones, which were cultivated for indicated periods; i.e., from 7 days as the early (E) stage, via several in-between cultivation periods including 33 days as the middle (M) stage, to 64 days as the late (L) stage. First, we carried out RT-PCR on these total RNAs using the above-mentioned specific primers IgM Hc, IgM Hs, IgM Hm plus IgM L, and HDAC2 plus β-actin as negative plus positive controls (Fig. 6). The levels of whole and secreted forms of IgM H-chain mRNA were dramatically increased at the early (E) stage in all of the tested four mutant clones. Thereafter, the increased levels of these two type mRNAs were gradually and dramatically decreased via the in-between cultivation periods in almost similar pattern in all of them and at the late (L) stage reached to comparable levels in DT40 cells. The certainly increased level of membrane-bound form of IgM H-chain mRNA at the early (E) stage was surely decreased during cultivation: the decrease being somewhat great in clone cl.2-1 or small in clones cl.2-2, cl.2-4 and cl.2-6. On the other hand, in all of the four mutant clones the mRNA level of IgM L-chain was slightly increased at the early (E) stage and thereafter slowly decreased during cultivation.

Gene expressions of PCAF, HDAC7 and HDAC9 change in different patterns in individual clones of HDAC2(-/-) mutants during continuous cultivation

Next, we carried out RT-PCR on the total RNAs, using appropriate primers specific for the above-mentioned altered chromatin modifying enzymes (Fig. 6). Remarkably, the changing patterns in gene expressions of PCAF, HDAC7 and HDAC9 were clearly distinct in individual clones of HDAC2(-/-) mutants during cultivation as follows. The mRNA level of PCAF, which was a very low in DT40 cells (W), was dramatically increased until the in-between periods (17 ~ 22 days) in four mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6). Thereafter, the increased mRNA level remained unchanged in clones cl.2-1, cl.2-2 and cl.2-4, but was dramatically decreased in clone cl.2-6. At the late (L) stage, the PCAF mRNA level was higher in clones cl.2-1, cl.2-2 and cl.2-4 than in DT40 cells, but in clone cl.2-6 it was almost similar to that in DT40 cells. The mRNA level of HDAC7, which was high in DT40 cells, showed almost similar changing pattern in all of the tested mutant clones during cultivation. That is, the mRNA level of HDAC7 in these four clones was obviously decreased at the early (E) stage but certainly increased until the in-between cultivation periods (17 ~ 27 days) and thereafter remained unchanged or very slowly decreased. Moreover, the mRNA level of HDAC7 at any cultivation stages in these mutant clones was lower than that in DT40 cells. The mRNA level of HDAC9, which was undetectable in DT40 cells, was gradually and certainly increased up to the in-between cultivation periods (17 ~ 22 days) in the four mutant clones, and thereafter the increased mRNA level was gradually decreased during cultivation. At the late (L) stage, the mRNA level of HDAC9 in clone cl.2-1 was extremely higher than that in DT40 cells, but in clones cl.2-2, cl.2-4 and cl.2-6 it was comparable to that in DT40 cells.

Gene expressions of Pax5, Aiolos, EBF1, OBF1, Blimp1, Ikaros, E2A and PU.1 change dramatically or moderately in distinct patterns in individual clones of HDAC2(-/-) mutants during continuous cultivation

Finally, we carried out RT-PCR on the total RNAs, using appropriate primers specific for the above-mentioned altered transcription factors (Fig. 6). Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, XBP-1 and OBF1 (but not Blimp1) genes were sufficiently or considerably expressed in DT40 cells (W). Surprisingly, however, gene expression patterns of these transcription factors (except XBP-1) were complicate and diverse in four individual clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) of HDAC2(-/-) mutants during cultivation as follows.

Changing patterns in mRNA levels of Pax5 and Aiolos during cultivation were very similar to each other within the same mutant clone but considerably different among these four individual mutant clones. In clone cl.2-1, mRNA levels of Pax5 and Aiolos were almost completely decreased at the early (E) stage, and thereafter remained unchanged or were very slightly increased until the late (L) stage during

cultivation. Remarkably, in three residual clones (cl.2-2, cl.2-4 and cl.2-6), mRNA levels of Pax5 and Aiolos were almost completely decreased at the early (E) stage, but thereafter these decreased mRNA levels were gradually and dramatically increased during cultivation. Namely, mRNA levels of Pax5 and Aiolos at the late (L) stage were approximately 20 ~ 40-folds or 80 ~ 120-folds of those of the two at the early (E) stage in clones cl.2-2 and cl.2-4 or clone cl.2-6. In addition, at the late (L) stage, mRNA levels of Pax5 and Aiolos in clones cl.2-2 and cl.2-4 or clone cl.2-6 were less than or comparable to those in DT40 cells. The mRNA level of EBF1 was almost completely decreased at the early (E) stage in four mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6), and thereafter showed almost similar changing pattern in the former three clones as a whole but a remarkably different changing pattern in the latter one clone during cultivation. That is, the decreased EBF1 mRNA level at the early (E) stage remained unchanged in clones cl.2-1, cl.2-2 and cl.2-4 until the late (L) stage to be undetectable or less than about 1% of that in DT40 cells, but gradually and dramatically increased in clone cl.2-6 until the late (L) stage to about 60% of that in DT40 cells. The mRNA level of OBF1 changed in almost similar pattern as a whole in three mutant clones cl.2-2, cl.2-4 and cl.2-6 during cultivation, but the changing pattern in clone cl.2-1 was obviously different from that in the former three clones. That is, in clones cl.2-2, cl.2-4 and cl.2-6, the mRNA level of OBF1 was decreased at the early (E) stage to about 30 ~ 40% of that in DT40 cells and thereafter slightly increased at the late (L) stage to about 60 ~ 100% of that in DT40 cells. By contrast, in clone cl.2-1, the mRNA level of OBF1 was gradually and dramatically decreased during cultivation until the late (L) stage to be less than 10% of that in DT40 cells.

In four mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6), the mRNA level of Blimp1, which was very low in DT40 cells, was dramatically elevated at the early (E) stage. Thereafter, the elevated Blimp1 mRNA level was slightly or dramatically reduced until the late (L) stage to about 50% of this elevated value in three clones (cl.2-1, cl.2-2 and cl.2-4) or to undetectable level in clone cl.2-6 as in DT40 cells. The mRNA level of Ikaros was slightly elevated at the early (E) stage in three clones (cl.2-1, cl.2-2 and cl.2-4) to about 150 ~ 200% of that in DT40 cells and thereafter remained unchanged until the late (L) stage, while did not change so much in clone cl.2-6 during cultivation. The mRNA level of E2A was elevated at the early (E) stage in the four clones to about 200 ~ 300% of that in DT40 cells and thereafter slowly reduced until the late (L) stage to about 100 ~ 200% of that in DT40 cells. The mRNA level of PU.1 was reduced at the early (E) stage in the four clones to about 30 ~ 50% of that in DT40 cells, and thereafter slightly elevated until the late (L) stage in clone cl.2-1 or in three clones cl.2-2, cl.2-4 and cl.2-6 to about 150% or 60% of that in DT40 cells. The XBP-1 mRNA level remained unchanged as a whole in the four clones during cultivation.

Growth rate of individual clones of HDAC2(-/-) mutants at the late stage of cultivation is different each other

As a typical cellular property, we finally studied growth rate of individual HDAC2(-/-) mutant clones at the late (L) cultivation stage (Fig. 7). The growth rate of mutant clones cl.2-1 and cl.2-4 was slightly but obviously slower than that of DT40 cells, but the growth rate of mutant clone cl.2-6 was almost similar to that of DT40 cells.

Discussion

In HDAC2-deficient DT40 mutant cells HDAC2(-/-), IgM H- and L-chains are dramatically and considerably accumulated at the early stage of continuous cultivation; i.e., soon after the generation (birth) by gene targeting techniques (Figs. 1, 2 and 4), because HDAC2 as a supervisor regulates indirectly gene expressions of these two immunoglobulin proteins through opposite control of Pax5, Aiolos, EBF1, OBF1, and E2A plus Ikaros gene expressions in wild type DT40 cells [41, 42, 48]. These results obtained from DT40 and HDAC2(-/-) mutant cells at the early cultivation stage are schematically shown in Figures 8 and 9, respectively. The majority of IgM H- and L-chains artificially accumulated in HDAC2(-/-) mutants exist as a native soluble form capable of building a high molecular weight complex with each other probably within endoplasmic reticulum [41]. The HDAC2 mediated regulatory mechanisms may not function any longer, and lacking of the mechanisms could be far superior to the capacity of secreting large amounts of these two immunoglobulin proteins in HDAC2(-/-) mutants [40, 41]. In addition, HDAC2(-/-) mutant cells exist rather as a morphologically aggregative (and also probably distorted) form at the early stage (Figs. 4 and 5), the real reason for which is still unknown. Anyhow, both of the accumulation of these two immunoglobulin proteins and aggregative form should be uncomfortable (or painful) environments for HDAC2(-/-) mutant cells themselves. Surprisingly, elevated protein levels of IgM H- and L-chains artificially accumulated at the early stage in HDAC2(-/-) mutants are gradually reduced during cultivation and at the late stage reached to comparable levels in DT40 cells (Figs. 2 and 4). In parallel with these changes, morphology of HDAC2(-/-) mutant cells also changes; i.e., the aggregative form at the early stage is altered during cultivation to the dispersive form at the late stage, like that of DT40 cells, and it must be comfortable (or peaceful) for both of DT40 and HDAC2(-/-) mutant cells (Fig. 5). Moreover, the morphology of HDAC2(-/-) mutants at the early stage and its changing pattern during cultivation are clearly different from those of Pax5(-) mutants [47].

Interestingly, as clearly presented in Figure 6, mRNA levels of various altered transcription factors (and chromatin modifying enzymes) showed following distinct changing patterns during cultivation in six individual clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) of HDAC2(-/-) mutants, in spite of almost similar changing pattern in protein and mRNA levels of IgM H- and L-chains (Figs. 1, 2, 3, 4 and 6) and also in cell morphology (Figs. 4 and 5). That is, remarkably, in clone cl.2-1, mRNA levels of Pax5, Aiolos and EBF1, all of which down-regulate gene expressions of IgM H- and L-chains [41, 42, 47, 48], are dramatically decreased at the early stage and remains unchanged until the late stage during

cultivation. By contrast, the mRNA level of OBF1, which probably up-regulates gene expressions of these two immunoglobulin proteins [61], is gradually and dramatically decreased until the late stage during cultivation. In clones cl.2-2 and cl.2-4 (and also cl.2-3 plus cl.2-5), mRNA levels of Pax5, Aiolos and EBF1 are dramatically decreased at the early stage, and thereafter mRNA levels of Pax5 and Aiolos are gradually increased until the late stage during cultivation but a very low mRNA level of EBF1 remains unchanged. On the other hand, the mRNA level of OBF1 insignificantly changes during cultivation. In clone cl.2-6, mRNA levels of Pax5, Aiolos and EBF1 are drastically decreased at the early stage and thereafter gradually and dramatically increased until the late stage during cultivation, whereas the change in that of OBF1 is insignificant. We would like to emphasize that any characteristic changes of HDAC2(-/-) (and also Pax5(-)) mutants should be more drastic just soon after their birth. Because, in this paper and our previous papers [42, 47] we collected the mutant cells at about 10 ~ 12 days after their birth (by gene targeting techniques) as the early (or first) stage and also their doubling times were about 12 hrs [40, 41, 49]; therefore, they should be populations of about 20 ~ 25 generations even at the first and early stages.

In summary, concerning manners for gene expressions of IgM H- and L-chains at the late cultivation stage, individual clones of HDAC2(-/-) mutants could be classified into following three distinct types. Clone cl.2-1 seems to be OBF1-dependent type and distinct from wild type DT40 cells in appearance. Clones cl.2-2, cl.2-3, cl.2-4 and cl.2-5 seem to be Pax5- and Aiolos-dependent type and slightly similar to DT40 cells in appearance. These four mutant clones should be major type, since four initially generated HDAC2(-/-) mutant clones roughly resembled them in several cellular characteristics [40, 41]. Clone cl.2-6 seems to be Pax5-, Aiolos- and EBF1-dependent type and most similar to DT40 cells in appearance. These results at the late cultivation stage in individual clones of HDAC2(-/-) mutants are schematically shown in Figure 10. On the other hand, alterations in the mRNA level of E2A, which up-regulates gene expressions of IgM H- and L-chains [41], must be such unrelated to the decreases in two immunoglobulin protein levels in HDAC2(-/-) mutants during cultivation, since its alteration was not remarkable in all of the six mutant clones (Figs. 3 and 6). Further, till quite recently, detailed participations of altered mRNA levels of PCAF, HDAC7, HDAC9 and Blimp1 in the decreases in protein and mRNA levels of IgM H- and L-chains in HDAC2(-/-) mutant clones during cultivation remain to be resolved, although slight deviations in those of IgM H- and L-chains were detected in both cases of PCAF- and GCN5-deficiencies [41]. Very recently, we reported that the IgM H-chain (but not L-chain) gene expression is slightly down-regulated by GCN5-deficiency [62], and the secretory immunoglobulin synthesis is suppressed by PCAF-deficiency [63]. In any case, the above-mentioned classification of individual clones of HDAC2(-/-) mutants must be partly supported by findings that the growth rate of mutant clone cl.2-6 or clones cl.2-1 and cl.2-4 (and probably cl.2-2, cl.2-3 plus cl.2-5) at the late stage was almost similar to or slightly but certainly different from that of DT40 cells (Fig. 7). Remarkably,

the classification of HDAC2(-/-) mutant clones based on altered transcription factors is coincident with that based on altered members of HATs and HDACs as mentioned above (Fig. 3). If additional independent clones of HDAC2(-/-) mutants are analyzed, other distinct types will be probably added as manners for gene expressions of IgM H- and L-chains, besides the above-mentioned three types. Moreover, such above-mentioned differences in gene expressions of numerous transcription factors and chromatin modifying enzymes suggest strongly that individual clones of HDAC2(-/-) mutants should be obviously distinct each other in some other cellular characteristics, although concrete data are not enough to support this possibility so far. Naturally, changes in gene expressions of various members of transcription factors, HATs and HDACs, and decreasing speeds of protein and mRNA levels of IgM H- and L-chains, and also cell morphology during cultivation were obviously different between HDAC2(-/-) and Pax5(-) mutants [47]. These results suggest that the above-mentioned three manners (and supposed ones) to decrease gene expressions of IgM H- and L-chains in HDAC2(-/-) mutants should be certainly different from those in Pax5(-) mutants.

In conclusion, individual clones of HDAC2(-/-) mutants each should possess the ability to gain the same and new cell functions in distinct manners via a lot of generations (cell divisions) during cultivation. Namely, the same and new cell functions mean not only excluding artificially accumulated IgM H- and L-chains but also ridding free from aggregative form, since they should be uncomfortable for the mutant cells themselves. These distinct manners should be definitely caused by diverse changes in gene expressions of transcription factors and chromatin modifying enzymes (such as Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, Blimp1, OBF1, PCAF, HDAC7, HDAC9 and others) in individual clones of HDAC2(-/-) mutants during cultivation. Of these altered factors and enzymes, Pax5, Aiolos, EBF1 and OBF1 should be most influential candidates participating in decreases in IgM H- and L-chain gene expressions, because the changing patterns in gene expressions of these four factors were anti-parallel or parallel with those in two immunoglobulin gene expressions in one or more of individual HDAC2(-/-) mutant clones. Based on these results, for manners to eliminate artificially accumulated IgM H- and L-chains in HDAC2(-/-) mutants, we propose a brief working hypothesis as follows (Fig. 11). First, the accumulation of IgM H- and L-chains in HDAC2(-/-) mutants should be recognized as uncomfortable environment change, and then putative signal(s) concerning it may be genome-widely transmitted to chromatin structure within nucleus, though the mechanism and machinery for these processes remain quite unknown. Successively, the environment change should induce alterations in chromatin structure of various transcription factor and chromatin modifying enzyme genes (such as Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, OBF1, Blimp1, XBP-1, Oct2, PCAF, HDAC7, HDAC9 and other genes), resulting in their altered transcription levels. The response(s) for the environment change should be continuously converged to chromatin structure of several particular factor and enzyme genes (Pax5, Aiolos, EBF1, OBF1, PCAF, HDAC7, HDAC9 and other genes) in individual mutant clones via a lot of generations

during cultivation, and thereby induce diverse alterations in their chromatin structure, resulting in their varied transcription levels. As a result, individual clones of HDAC2(-/-) mutants gain the same and new cell functions to exclude accumulated IgM H- and L-chains based on their decreased gene expressions through varied gene expressions of particular transcription factors (Pax5, Aiolos, EBF1, OBF1, etc.) in different manners during cultivation. Therefore, concerning this working hypothesis on exclusion of artificially accumulated IgM H- and L-chains, one of the most interesting subjects is elucidation of distinct manners for gene expressions of these particular transcription factors in individual clones of HDAC2(-/-) mutants via a lot of generations (cell divisions) during continuous cultivation.

Acknowledgments

The authors are grateful to Drs. A. Sawaguchi and T. Suganuma for electron microscopy and immuno-electron microscopy study. And the authors thank Dr. H. Kikuchi for microscopy study.

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Figure legends

Figure 1. Genomic organization of chicken HDAC2 gene, generation of HDAC2(-/-) DT40 mutants and alterations in protein and mRNA levels of IgM H- and L-chains in HDAC2(-/-) mutant clones during cultivation

A. Schematic representation of chicken HDAC2 genomic locus (top) with enlarged drawing of its intact allele (middle) and targeted alleles (two bottoms). Locations of exons are indicated by solid boxes with appropriate designations 1 to 16. White boxes indicate drug resistance cassettes (MerCreMer and hyg). Location of probe HDAC2 is indicated by a gray box. Only relevant restriction sites are indicated. Possible relevant fragments obtained from BamHI and EcoRV digestions are shown with their lengths in kb.

B. Southern blotting of homologous recombination event. Genomic DNAs were prepared from DT40, one heterozygous mutant clone cl.2(-/+) and six homozygous mutant clones cl.2-1(-/-), cl.2-2(-/-), cl.2-3(-/-), cl.2-4(-/-), cl.2-5(-/-) and cl.2-6(-/-). BamHI and EcoRV fragments were analyzed with probe HDAC2.

C. Western blotting. Total cellular proteins were prepared from six HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) at the early (E; 3 days), middle (M; 29 days) and late (L; 53 days) stages of cultivation and DT40 cells (W). Aliquots of cell extracts were subjected to 12% SDS-PAGE and transferred to membrane filters. Proteins were detected with anti-chicken IgM L-chain and H-chain antibodies. IgM H and IgM L, respectively, indicate IgM H-chain and IgM L-chain (as two bands), which are represented after cutting. Chicken β-actin was finally detected as control on the same membrane filters.

D. RT-PCR. Total RNAs were extracted from six HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) at the early (E; 7 days), middle (M; 33 days) and late (L; 64 days) stages of cultivation and DT40 cells (W). RT-PCR was performed on equal amounts of total RNAs, using appropriate primers for whole IgM H-chain mRNA (IgM Hc), its secreted form (IgM Hs), its membrane-bound form (IgM Hm) and IgM L-chain mRNA (IgM L). HDAC2 and chicken β-actin were used as negative and positive controls, respectively.

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

Total cellular proteins were prepared from HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) at indicated cultivation periods, including the early (E; 3 days), middle (M; 29 days) and late (L; 53 days) stages 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 filters. Proteins were first detected with anti-chicken IgM L-chain antiserum that cross-reacts with IgM H-chain.

Subsequently, after de-staining, those were detected with anti-chicken IgM H-chain antiserum, followed by detection of β-actin as in Figure 1. Protein bands were cut off and represented separately. Upper and lower bands indicated by IgM H correspond to IgM H-chain detected by antibodies for IgM H- and L-chains, respectively. IgM L-chain (indicated by IgM L) was detected as two bands.

Figure 3. Alterations in gene expressions of HDACs, HATs and transcription factors in individual clones of HDAC2(-/-) DT40 mutants during cultivation

RT-PCR was performed on total RNAs identical with those in Figure 1D, using appropriate primers for members of HDACs and HATs (A) and transcription factors (B).

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

Four HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) collected at the early (E; ~5 days) and late (L; ~60 days) stages of cultivation and DT40 cells (W) were fixed. Electron microscopy (upper panels) and immuno-electron microscopy using anti-chicken IgM H-chain antiserum (lower panels) were carried out. Dense cytoplasmic fractions due to accumulated IgM H-chain proteins were observed only at the early (E) stage in the four mutant clones (E in upper panels). Positive signals for IgM H-chain proteins were observed only at the early (E) stage in these mutant clones (E in lower panels).

Figure 5. Alterations of morphology of individual clones of HDAC2(-/-) DT40 mutants during cultivation

Microscopy was performed on four HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) at the early (E; ~5 days) and late (L; ~60 days) stages of cultivation and DT40 cells (W) in several different ranges of vision. The different ranges of vision within microscopy are represented. Aggregative form was observed only at the early (E) stage in these mutant clones.

Figure 6. Alterations in gene expressions of IgM H- and L-chains, particular members of HATs, HDACs and transcription factors in individual clones of HDAC2(-/-) DT40 mutants during cultivation

Total RNAs were extracted from four HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) at indicated cultivation periods, including the early (E; 7 days), middle (M; 33 days) and late (L; 64 days) stages and DT40 cells (W). RT-PCR was performed on total RNAs, using appropriate primers for IgM H- and L-chains, and particular members of HATs and HDACs, and transcription factors, most of which were altered in Figures 1D and 3.

Figure 7. Growth rate of individual clones of HDAC2(-/-) DT40 mutants

HDAC2(-/-) mutant clones (cl.2-1, cl.2-4 and cl.2-6) and DT40 cells (WT) were grown, and cell numbers were determined at indicated times nearby the late cultivation stage. The numbers are plotted on a log phase. The values are averages for three independent experiments. Symbols for these clones are shown in the figure.

Figure 8. A model for role of HDAC2 as a supervisor in all-inclusive control of gene expressions of IgM H- and L-chains through opposite control of gene expressions of Pax5, Aiolos, EBF1, OBF1, Ikaros and E2A in wild-type DT40 cells

Figure 9. Models for roles of Pax5, Aiolos and EBF1 in control of gene expressions of IgM H- and L-chains in all clones of HDAC2(-/-) DT40 mutants at early stage of cultivation

Figure 10. Models for roles of Pax5, Aiolos, EBF1 and OBF1 in control of gene expressions of IgM H- and L-chains in individual clones of HDAC2(-/-) DT40 mutants at late stage of cultivation

Figure 11. A proposed model for manners to exclude IgM H- and L-chains accumulated in HDAC2(-/-) DT40 mutant clones during cultivation