IgM H- and L-chains artificially and excessively accumulated in HDAC2(-/-) DT40 mutants are gradually and dramatically reduced in distinct ways in individual mutant clones through various generations during continuous cultivation[**]

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Abstract

We newly generated histone deacetylase-2 (HDAC2)-deficient DT40 mutants HDAC2(-/-) and analyzed their characteristics at various intervals during continuous cultivation. In six independent clones cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and 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 later stage reached comparable levels as 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 later 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 later 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 ways of gene expressions of IgM H- and L-chains exist at the later stage in these six individual HDAC2(-/-) clones; i.e., clone cl.2-1 seems the OBF1-dependent type, clones cl.2-2, cl.2-3, cl.2-4 and cl.2-5 seem the Pax5- and Aiolos-dependent type, and clone cl.2-6 seems the Pax5-, Aiolos- and EBF1-dependent type. Based on these results, we proposed a hypothesis that individual clones of HDAC2(-/-) mutants possess the ability to gain new cell function to exclude excessively accumulated IgM H- and L-chains (as an abnormal and uncomfortable environment change) based on their decreased gene expressions through distinct ways depending on alterations in gene expressions of Pax5, Aiolos, EBF1, OBF1 and others through various generations during continuous cultivation.

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 for 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 ways for expression of cell functions in eukaryotes. For the last several decades, mechanisms to modulate the chromatin structure with epigenetic modifications, such as

acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation and others, have been intensively studied in a variety of research fields of life science. Of these epigenetic modifications, acetylation and deacetylation of specific Lys residues of core histones (H2A, H2B, H3 and H4) 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 the chromatin structure have been vigorously continued without interruptions in more diverse life science fields, e.g., gene expression/transcription, DNA replication, differentiation, development, memory, pluri-potency, clinical medicine and others [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 systematically studied in vivo roles of a large number of specific 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 two 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 specific 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 the regulation of gene expressions of IgM H- and L-chains, we 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 generated Pax5-deficient mutant cell line Pax5(-), devoid of a single Pax5 allele [47] existing on Z sex chromosome that is monosomy in chickens (USCS Genome Browser data base) and Ikaros-down mutant cell line Ikaros(-/-/+), devoid of two of three Ikaros alleles (our unpublished data) existing 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 accidentally noticed following remarkable phenomena [42]. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) showed that in HDAC2(-/-) mutants IgM H- and L-chains are dramatically elevated at the early stage of cultivation and thereafter gradually reduced during cultivation and at the later stage reach comparable levels as in DT40 cells, whereas alterations in cellular levels of most of other major proteins are

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 are clearly accumulated at the early stage and thereafter reduced at the later stage to almost the same level as in DT40 cells. Reverse transcription-polymerase chain reaction (RT-PCR) using primers IgM Hc and IgM Hs showed that whole and secreted forms of IgM H-chain mRNA are dramatically increased at the early stage, and thereafter gradually decreased during cultivation and at the later stage reach very close levels as in DT40 cells. These results, together, indicated that in HDAC2(-/-) mutants IgM H- and L-chains are dramatically and considerably accumulated depending on their increased gene expressions at the early stage of cultivation, and gradually reduced depending on their decreased gene expressions during cultivation and at the later stage finally reach comparable levels as in DT40 cells. Remarkably, RT-PCR, using appropriate primers specific for various genes encoding respective members of HDACs, HATs and transcription factors, showed that gene expressions of PCAF, HDAC7, HDAC9, EBF1, Pax5, E2A, Aiolos and others separately change in different ways 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 are gradually increased during cultivation, whereas insignificant changes are observed for most of examined Lys residues of other core histones. Furthermore, 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 (GenBank accession number: 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 the chromatin structure surrounding the proximal ~2.0-kb 5'-upstream region of the Pax5 gene are decreased at the early stage in HDAC2(-/-) mutants and at the later stage increased to comparable levels as in DT40 cells. Of these newly obtained results from the initially generated HDAC2(-/-) mutants, it should be worth of special mention that IgM H- and L-chains artificially and excessively accumulated at the early cultivation stage are diminished depending on their decreased gene expressions, which is mainly attributed to alterations in gene expressions of the above-mentioned specific transcription factors during cultivation [42]. Besides, in Pax5(-) mutants the two immunoglobulin proteins artificially and dramatically accumulated at the early stage are more rapidly reduced depending on their decreased gene expressions, and this is probably attributed to alterations in gene expressions of some of specific transcription factors and chromatin-modifying enzymes during cultivation [47].

In this study, to closely clarify the mechanism that causes decreases in gene expressions of IgM H- and L-chains during cultivation, 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 later stage in almost similar changing pattern in all of six tested independent clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) of HDAC2(-/-) mutants. By contrast, surprisingly, these individual HDAC2(-/-) clones 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 depending on their decreased gene expressions, which were mainly attributed to varied alterations in respective gene expressions of Pax5, Aiolos, EBF1, OBF1 and others during cultivation. That is, three distinct ways of gene expressions of IgM H- and L-chains exist at the later stage; i.e., clone cl.2-1 seems to be the OBF1-dependent type, clones cl.2-2, cl.2-3, cl.2-4 and cl.2-5 seem to be the Pax5- and Aiolos-dependent type, and clone cl.2-6 seems to be the Pax5-, Aiolos- and EBF1-dependent type. Based on these results, we proposed models for the role of HDAC2 in diminishing IgM H- and L-chains excessively accumulated in individual clones of HDAC2(-/-) mutants by means of distinct ways to change gene expressions of Pax5, Aiolos, EBF1, OBF1 and others, probably depending on their chromatin conformation changes through various generations during continuous cultivation.

Methods

Cell cultures

HDAC2(-/-) DT40 mutant cells, which were 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 later (L) stages of cultivation. In this article, the cultivation stages and periods were practically counted from the first day of cultivation from the stock at -80 °C. DT40 (W) was used as a control. Cell numbers were counted at indicated times nearby the later 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, using appropriate restriction enzymes (SpeI plus SphI, and SphI plus BamHI), 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, 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. The probe HDAC2 was the 0.4-kb BamHI/SpeI fragment, corresponding to the 5'-outer side of 5'-arm (i.e., 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 later (L; 64 days) cultivation stages and from DT40 cells (W). RT-PCR was carried out using appropriate sense and anti-sense primers listed in Table I and also in 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 later (L; 53 days) cultivation stages, and from 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 plus goat anti-chicken IgM H-chain antiserum (as primary antibodies), and finally antiserum for β-actin as a 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 anti-chicken IgM H-chain antiserum were carried out essentially as described [42, 43, 47, 59, 60]. Briefly, post-embedding immunocytochemistry using immune-gold labeling was carried out as follows. Four HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) at the early (E; ~5 days) and later (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 post-fixed 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 a 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, all of which had 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, the endogenous ~4.0-kb band derived from two intact alleles disappeared and the ~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 all of 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(-/-) DT40 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) at the early (E; 3 days), middle (M; 29 days) and later (L; 53 days) stages of cultivation (which were regarded as cultivation periods of ~5 days, ~30 days and ~50 to 60 days, respectively), and from DT40 cells (W; wild-type) as a control (Fig. 1C). As mentioned above, these three cultivation stages were practically counted from the first day of cultivation from the stock at -80 °C. As a 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 these mutant clones during cultivation. That is, the IgM H-chain level was moderately decreased at the middle (M) stage and dramatically decreased at the later (L) stage to comparable level as 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 later (L) stage to a level very close to that 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), 33 days as the middle (M), and 64 days as the later (L) stages, and from DT40 cells (W) as a 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 a control, β-actin was used, since its mRNA level at the early (E), middle (M) and later (L) stages in the six mutant clones was usually kept constant in the range of approximately 85 ~ 110% of the 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 later (L) stage to almost the same levels as 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 through the middle (M) to later (L) stages to about 50% in mutant clone cl.2-1. In addition, in all of the six mutant clones the level of IgM L-chain mRNA slightly changed 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 almost similar pattern in individual clones of HDAC2(-/-) DT40 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 seem to be very interesting, surprising and important phenomena. To confirm these findings, we carried out Western blotting at shorter intervals in further detail, 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 later (L; 53 days) stages, and from DT40 cells (W) (Fig. 2). As a 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 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, and thereafter drastically decreased throughout the in-between cultivation periods including the middle (M) stage until the later (L) stage and reached almost the same level as in

DT40 cells. 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 and reached almost the same level as 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 gradually decreased throughout the in-between cultivation periods including the middle (M) stage until the later (L) stage and reached almost the same level as 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 six clones of HDAC2(-/-) mutants were surely and dramatically decreased in almost similar changing pattern during cultivation.

Gene expressions of various members of chromatin modifying enzymes (HDACs and HATs) and transcription factors change in individual clones of HDAC2(-/-) DT40 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 respective genes encoding various 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), all of which were cultivated for 7, 33 and 64 days as the early (E), middle (M) and later (L) stages, and from DT40 cells (W) (Fig. 3A). In these six mutant clones, mRNA levels of HDAC7, HDAC9 and PCAF significantly changed 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, the 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.

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 respective genes encoding various members of transcription factors (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 changed factors, the six mutant clones could also be 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, in agreement with the above-mentioned classification based on the changing patterns for members of HDACs and HATs.

Because four mutant clones cl.2-2, cl.2-3, cl.2-4 and cl.2-5 had a great resemblance in many cellular

properties with each other as mentioned above, we decided clones cl.2-2 and cl.2-4 to the representative of the four mutant clones. Hereafter, the changing patterns in gene expressions of the altered specific transcription factors and chromatin-modifying enzymes and also 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(-/-) DT40 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 at the later (L) stage obviously reduced to almost the same level as in DT40 cells (W) [42]. To confirm these findings and to explore whether newly (i.e., secondly) generated HDAC2(-/-) mutant cells morphologically change during cultivation, we first examined the morphology of the four mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) and DT40 cells as a control. Electron microscopy showed that all of these four mutant clones were observed in a somewhat distorted form at the early (E) stage but at the later (L) stage were in a 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 later (L) stage, similar to that 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 four mutant clones, and thereafter most of the accumulated proteins disappeared at the later (L) stage, like in DT40 cells (Fig. 4; lower panels). These results roughly but surely agreed with those observed 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 later (L) cultivation stages and on DT40 cells (Fig. 5). Interestingly, these four mutant clones were morphologically observed to be in an aggregative form at the early (E) stage but in a dispersive form at the later (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(-/-) DT40 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), changed in the six tested HDAC2(-/-) mutant clones during cultivation. We very closely examined the changing patterns in gene expressions of IgM H- and L-chains and the changed 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., for 7 days as the early (E) stage, several in-between

cultivation periods including 33 days as the middle (M) stage, and 64 days as the later (L) stage. First, we carried out RT-PCR on the total RNAs, using the above-mentioned specific primers IgM Hc, IgM Hs, IgM Hm and IgM L (Fig. 6). HDAC2 plus β-actin were used as negative plus positive controls. 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 dramatically decreased through the in-between cultivation periods in almost similar pattern in all of them, and at the later (L) stage reached 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 huge 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(-/-) DT40 mutants during continuous cultivation

Next, we carried out RT-PCR on the total RNAs, using appropriate primers specific for the above-mentioned changed chromatin-modifying enzymes (Fig. 6). Remarkably, the changing patterns in gene expressions of PCAF, HDAC7 and HDAC9 were clearly distinct among 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 later (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 these four mutant clones during cultivation. That is, the mRNA level of HDAC7 in the 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 the four mutant clones was lower than that in DT40 cells. The mRNA level of HDAC9, which was undetectable in DT40 cells, was gradually but 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 later (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 dramatically or moderately change in distinct patterns in individual clones of HDAC2(-/-) DT40 mutants during

continuous cultivation

Finally, we carried out RT-PCR on the total RNAs, using appropriate primers specific for the above-mentioned changed 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, gene expression patterns of these transcription factors (except XBP-1) changed complicatedly and diversely 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 later (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 two decreased mRNA levels were dramatically increased during cultivation. Precisely, mRNA levels of Pax5 and Aiolos at the later (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 later (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 first three clones as a whole but a remarkably different changing pattern in the last 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 later (L) stage to be undetectable or less than about 1% of that in DT40 cells, but dramatically increased in clone cl.2-6 until the later (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 first 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 later (L) stage to about 60-100% of that in DT40 cells. By contrast, in clone cl.2-1, the mRNA level of OBF1 was dramatically decreased during cultivation until the later (L) stage to 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 later (L) stage to about 50% of the elevated value in three clones cl.2-1, cl.2-2 and cl.2-4 or to an 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 later (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 later (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 later (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(-/-) DT40 mutants at the later stage of cultivation is different from each other

As a typical cellular property, we finally studied growth rate of individual HDAC2(-/-) mutant clones at the later (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 their generation (birth) by gene targeting techniques (Figs. 1, 2 and 4), because HDAC2 as a supervisor indirectly regulates gene expressions of the 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 somewhat distorted) form at the early stage (Figs. 4 and 5), the real reason for which is still unknown. Anyhow, the accumulation of these two immunoglobulin proteins and the aggregative form should be abnormal, uncomfortable (or painful) environments to HDAC2(-/-) mutant cells themselves. Surprisingly, the elevated protein levels of IgM H- and L-chains artificially accumulated at the early stage in HDAC2(-/-) mutants are dramatically reduced during cultivation and at the later stage reach comparable levels as in DT40 cells (Figs. 2 and 4). In parallel with the change, the morphology of

HDAC2(-/-) mutant cells also changes; i.e., the aggregative form at the early stage changes during cultivation to the dispersive form at the later stage, like that of DT40 cells, and the dispersive form must be normal and comfortable (or peaceful) state 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 changed 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 later stage during cultivation. By contrast, the mRNA level of OBF1, which probably up-regulates gene expressions of these two immunoglobulin proteins [61], is dramatically decreased until the later 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 and dramatically increased until the later stage during cultivation but the mRNA level of EBF1 remains unchanged during cultivation as it was very low. 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 dramatically increased until the later stage during cultivation, whereas the change in that of OBF1 is insignificant. By the way, we would like to emphasize that any characteristic changes of HDAC2(-/-) (and also Pax5(-)) mutants should be more drastic just soon after their birth (by gene targeting techniques). The reasons are as follows. For instance, in this study even HDAC2(-/-) mutant cells at the early stage (~3 days-cultivation from the stock at -80 °C) should be populations around 30-32 generations after their birth. Because, prior to the stock they were already cultivated on agar plate and continuously in liquid medium for at least 12-13 days and also their doubling times were reported to be about 12 hrs [40, 41, 49].

In summary, with regard to the ways of gene expressions of IgM H- and L-chains at the later cultivation stage, individual clones of HDAC2(-/-) mutants could be classified into following three distinct types. Clone cl.2-1 seems to be the OBF1-dependent type and distinct from wild-type DT40 cells in appearance. Therefore, clone cl.2-1 resembles Pax5(-) mutants in the way to decrease the accumulated immunoglobulin proteins during cultivation in appearance, since the gene expression of OBF1 (with changes in gene expressions of Aiolos, EBF1, PCAF, HDAC7 and HDAC9) is dramatically decreased in almost similar pattern in both clone cl.2-1 (Fig. 4) and Pax5(-) [47]. Clones cl.2-2, cl.2-3, cl.2-4 and cl.2-5 seem to be the 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 the Pax5-, Aiolos- and EBF1-dependent type and most similar to DT40 cells in appearance. These results at the later 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 unrelated to the decreases in the 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 changed 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 are detected in both cases of the 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 the GCN5-deficiency [62], and the secretory immunoglobulin synthesis is suppressed by the PCAF-deficiency [63]. In any case, the above-mentioned classification of individual clones of HDAC2(-/-) mutants must be partly supported by the 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 later stage is almost similar to or slightly but certainly different from that of DT40 cells (Fig. 7). Remarkably, the classification of HDAC2(-/-) mutant clones based on alterations in gene expressions of specific transcription factors is coincident with that based on alterations in gene expressions of specific 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 ways of 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 strongly suggest that individual clones of HDAC2(-/-) mutants are obviously distinct from each other in some other cellular characteristics, although concrete data are not enough to support this possibility so far. Naturally, alterations in the gene expressions of various members of transcription factors, HATs and HDACs, and in the decreasing speeds of protein and mRNA levels of IgM H- and L-chains, and also in the cell morphology during cultivation were obviously different between HDAC2(-/-) and Pax5(-) mutants [47]. These results suggest that the above-mentioned three ways (and supposed ones) that 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 should possess the ability to gain the same and new cell functions in distinct ways through various generations (cell divisions) during continuous cultivation. Namely, the same and new cell functions mean not only diminishing artificially accumulated IgM H- and L-chains but also ridding free from the aggregative form, since they should be

abnormal and uncomfortable for the mutant cells themselves. These distinct ways should be definitely caused by diverse alterations 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 the 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 are anti-parallel or parallel with those in two immunoglobulin gene expressions in one or more of individual HDAC2(-/-) mutant clones. Based on these results, as mechanisms to diminish artificially accumulated IgM H- and L-chains in HDAC2(-/-) mutants, we proposed a brief working hypothesis as follows (Fig. 11). First, the accumulation of IgM H- and L-chains in HDAC2(-/-) mutants should be recognized as an abnormal and uncomfortable environment change, and then putative signal(s) concerning it may be genome-widely transmitted to the chromatin structure within the nucleus, whereas the mechanism and machinery for the processes remain quite unknown. Successively, the environment change should induce alterations in the 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 others), resulting in their altered transcription levels. The response(s) for the environment change should be continuously converged to the chromatin structure of several specific factor and enzyme genes (Pax5, Aiolos, EBF1, OBF1, PCAF, HDAC7, HDAC9 and others) in individual mutant clones through various generations during cultivation, and thereby induce diverse alterations in their chromatin structure, resulting in their varied gene expression levels. As a result, individual clones of HDAC2(-/-) mutants gain the same and new cell function to exclude accumulated IgM H- and L-chains depending on their decreased gene expressions through varied gene expressions of specific transcription factors (Pax5, Aiolos, EBF1, OBF1, etc.) in different ways 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 ways for gene expressions of these specific transcription factors in individual clones of HDAC2(-/-) mutants through various generations (cell divisions) during continuous cultivation.

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Conflict of interest statement

There are no conflicts of interest.

Postscript

The studies in the article were reviewed in Ref. 64.

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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(-/-) DT40 mutant clones during continuous 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 1-16. 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 later (L; 53 days) stages of cultivation and from 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 presented after cutting. Chicken β-actin was finally detected as a 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 later (L; 64 days) stages of cultivation and from 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) plus its membrane-bound form (IgM Hm), and IgM L-chain mRNA (IgM L), respectively. 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 continuous 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 later (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 presented separately. Upper and

lower bands (indicated by IgM H) correspond to IgM H-chain detected by antibodies for IgM H- and L-chains, respectively. Two forms of IgM L-chain (indicated by IgM L) were detected as two bands.

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

RT-PCR was performed on total RNAs identical with those in Figure 1D, using appropriate primers for various 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 continuous cultivation.

Four HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) at the early (E; ~5 days) and later (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 four mutant clones (E in lower panels).

Figure 5. Alterations of morphology of individual clones of HDAC2(-/-) DT40 mutants during continuous 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 later (L; ~60 days) stages of cultivation and on DT40 cells (W) in several different ranges of vision. The different ranges of vision within microscopy are presented. The aggregative form was observed only at the early (E) stage in these four mutant clones.

Figure 6. Alterations in gene expressions of IgM H- and L-chains, various members of HATs, HDACs and transcription factors in individual clones of HDAC2(-/-) DT40 mutants during continuous 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 later (L; 64 days) stages and from DT40 cells (W). RT-PCR was performed on total RNAs, using appropriate primers for IgM H- and L-chains, and various members of HATs, HDACs, and transcription factors, most of which changed 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 later cultivation stage. The numbers are plotted on a log phase. The values are averages for three independent experiments. Symbols for these three clones and DT40 cells 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 later stage of cultivation.

Figure 11. Proposed models for ways to exclude IgM H- and L-chains accumulated in HDAC2(-/-) DT40 mutant clones during continuous cultivation.