Chapter 4

Artificially 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

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SUMMARY

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, 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 certainly reduced in almost similar changing pattern and at the late stage reached to comparable levels in DT40 cells. By contrast, mRNA levels of various transcription factors and chromatin modifying enzymes showed remarkably distinct changing patterns in the six HDAC2(-/-) mutant clones during cultivation. In clone cl.2-1, mRNA levels 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, mRNA levels of Pax5, Aiolos and EBF1 were almost completely decreased at the early stage, and thereafter mRNA levels of Pax5 and Aiolos were obviously increased until the late stage but that of EBF1 remained unchanged during cultivation. In clone cl.2-6, those 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 cultivation stage in these 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 HDAC2(-/-) mutant cells possess the ability for gain of new cell function in distinct manners to exclude excessively accumulated IgM H- and L-chains as uncomfortable environment change based on decreases in their gene expressions through alterations in transcriptions of several particular transcription factor genes based on their chromatin conformation changes via a lot of generations.

INTRODUCTION

During the process of studies on characteristics of initially generated HDAC2-deficient DT40 mutants HDAC2(-/-) [1, 2], which were cultivated for different periods, we casually noticed following interesting phenomena [Chap. 2]. 2D-PAGE showed that IgM H- and L-chains were dramatically elevated at the early stage of cultivation in HDAC2(-/-), and thereafter gradually decreased during cultivation and at the late stage reached to comparable levels in DT40 cells. On the other hand, 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

protein was 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 plus IgM Hs showed that whole and secreted forms of IgM H-chain mRNA were dramatically increased at the early stage, and thereafter gradually reduced during cultivation and at the late stage reached to very close levels in DT40 cells. These results, together, indicated not only that in HDAC2(-/-) IgM H- and L-chains are dramatically and considerably accumulated based on their increased gene expressions at the early stage of cultivation, but also that these two accumulated proteins are 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 chicken chromatin modifying enzymes (HDACs and HATs) and transcription factors, showed that gene expressions of PCAF, HDAC7, HDAC9, EBF1, Pax5, E2A, Aiolos and others were separately altered in different manners 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, although insignificant changes were observed for most of examined Lys residues of other core histones. Further, 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 thereafter increased at the late stage to comparable levels in DT40 cells.

In parallel and/or subsequently, to know respective roles of these altered transcription factors and others in regulations 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 [2-6]. In addition, we generated Pax5-deficient mutant cell line Pax5(-), devoid of the Pax5 gene [Chap. 3] located on Z chromosome that is monosomy 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 in chickens (USCS Genome Browser data base). Analyses of these resultant mutants revealed that Pax5, EBF1, Aiolos and Ikaros down-regulate transcriptions of IgM H- and L-chain genes, and E2A up-regulates transcriptions of these two immunoglobulin genes [2].

These results obtained from the above-mentioned DT40 mutant cell lines each devoid of HDAC2 or several transcription factors, revealed that HDAC2 indirectly regulates transcriptions of IgM H- and L-chain genes through opposite regulations of gene expressions of Pax5, EBF1, Aiolos plus Ikaros, and E2A [2, 7, Chaps. 2 and 3]. In addition, it should be worth of special mention that artificially accumulated two immunoglobulin proteins in HDAC2(-/-) are diminished based on their decreased gene expressions, attributed to altered gene expressions of the above-mentioned particular transcription factors (and possibly other transcription factors and chromatin modifying enzymes) during cultivation [Chap. 2].

In this study, to clarify manners for gene expressions of IgM H- and L-chains in further detail, we newly generated homozygous HDAC2-deficient DT40 mutants HDAC2(-/-) by gene targeting techniques [1, 8-18, Chap. 3]. Remarkably, protein and mRNA levels of IgM H- and L-chains were dramatically and considerably increased at the early stage of cultivation, and thereafter drastically and gradually decreased until the late stage in almost similar changing pattern in all of six independent clones of HDAC2(-/-) mutants tested. Surprisingly, 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. These results revealed that artificially accumulated IgM H- and L-chains at the early cultivation stage in HDAC2(-/-) were diminished based on their decreased gene expressions, attributed to alterations in gene expressions of some of these particular transcription factors and chromatin modifying enzymes in different manners in individual mutant clones during cultivation. Based on these results, we propose a hypothesis that HDAC2(-/-) mutant cells have the ability to gain new cell function in distinct manners to exclude artificially accumulated IgM H- and L-chains as newly encountered uncomfortable environment change through altered gene expressions of several particular transcription factors based on their chromatin conformation changes during cultivation.

RESULTS

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

As shown in Figure 4-1A, genomic DNA of chicken HDAC2 consists of 16 exons, different from 14 exons reported in our previous paper [1]. We first generated homozygous HDAC2-deficient DT40 mutant cells HDAC2(-/-) devoid of two HDAC2 alleles by gene targeting techniques as described [1, 8-18, Chap. 3]. To eliminate effects of drug resistant genes in targeting vectors, we newly constructed two targeting vectors containing MerCreMer/bleo and hyg, which were different from those used previously [1]. Then, we sequentially introduced these two targeting vectors into DT40 cells derived from chicken B lymphocyte cells [16-18]. 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 4-1B. 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. In addition, as expected, RT-PCR using primers specific for HDAC2 showed no band for mRNA originated from the intact HDAC2 gene in six clones (Fig. 4-1D), confirming that these six clones (and also 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. 4-1C). These three cultivation stages and other periods were practically counted from the first stock day, although any 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. 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 of cultivation in these six HDAC2(-/-) clones was dramatically increased compared with that in DT40 cells. Thereafter, the accumulated protein level was gradually decreased in all of the tested mutant clones during cultivation; i.e., the protein 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 accumulated protein level was gradually decreased in all of the tested mutant clones during cultivation; i.e., the protein level was moderately decreased at the middle (M) stage and considerably decreased at the late (L) stage to very close level in DT40 cells (W).

Next, to know whether mRNA levels of IgM H- and L-chains change in HDAC2(-/-) during cultivation, we carried out RT-PCR using appropriate primers specific for chicken IgM H- and L-chains on total RNAs prepared from 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. 4-1D). As described previously [1, 2, Chaps. 2 and 3], 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 was usually kept constant in the range of approximately 85 ~ 110% of wild-type value (in DT40 cells) at the early (E; 7 days), middle (M; 33 days) and late (L; 64 days) stages in these six mutant clones. The levels of whole and secreted forms of IgM H-chain mRNA were drastically increased at the early (E) stage in all of six mutant clones compared with those in DT40 cells. Thereafter, accumulated 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 reduced via the middle (M) to late (L) stages to be about 50% in mutant clone cl.2-1. In addition, in all of six mutant clones the level of IgM L-chain mRNA changed very slightly or remained unchanged at any cultivation stages.

Accumulated protein levels of IgM H- and L-chains caused by the HDAC2-deficiency are dramatically and gradually reduced in HDAC2(-/-) mutants during continuous cultivation

The above-mentioned results that protein and mRNA levels of IgM H- and L-chains in HDAC2(-/-) dramatically changed during cultivation must be very interesting/surprising/important phenomena. To confirm these findings, we carried out Western blotting more minutely at shorter intervals, using antibody specific for chicken IgM H-chain and that considerably specific for chicken IgM L-chain that cross-reacts with IgM H-chain. Total cellular proteins were prepared from six mutant clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) cultivated for several indicated periods, including the early (E; 3 days), middle (M; 29 days) and late (L; 53 days) stages and DT40 cells (W) (Fig. 4-2). As control, β-actin was finally detected using its specific antibody on the same membrane filters. As was 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 in all of six mutant clones at the early (E) stage, but also that the accumulated protein level was drastically decreased until the late (L) stage to almost the same level in DT40 cells (W) via indicated 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 accumulated 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) in six mutant clones was certainly increased at the early (E) stage, and thereafter the accumulated protein level was gradually decreased via indicated 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 accumulated protein (and mRNA) levels of IgM H- and L-chains at the early (E) stage in all of the tested clones of HDAC2(-/-) mutants are surely/dramatically reduced during cultivation.

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

To see whether mRNA levels of chromatin modifying enzymes change in HDAC2(-/-) during cultivation, we carried out RT-PCR, using appropriate primers specific for various chicken HDACs and HATs, on total RNAs prepared from six 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. 4-3A). In these six mutant clones, the 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 the remaining 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(-/-) during cultivation, we carried out RT-PCR using appropriate primers specific for various chicken transcription factors on total RNAs mentioned above (Fig. 4-3B). In one or more of six mutant clones, the 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, 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 the remaining clone cl.2-6, agreed with the above-mentioned classification based on the changing patterns for HDACs and HATs. The changing patterns in gene expressions of these particular transcription factors and some other properties of four mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 will be shown in detail later.

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

Immuno-electron microscopy of initially generated HDAC2(-/-) mutant cells [1], using antibody specific for chicken IgM H-chain, showed that the immunoglobulin protein was clearly accumulated at the early (E; ~5 days) stage and thereafter obviously reduced at the late (L; ~60 days) stage to almost the same level in DT40 cells [Chap. 2]. To conform these findings and to explore whether HDAC2(-/-) morphologically changes 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 these mutant clones were observed to be somewhat distorted form at the early (E) stage and at the late (L) stage to be smooth form, like that of DT40 cells (Fig. 4-4; 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 in all of these mutant clones, 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 the IgM H-chain proteins were certainly accumulated at the early (E) stage, and thereafter most of the accumulated proteins disappeared at the late (L) stage, like in DT40 cells (Fig. 4-4; lower panels). These results roughly but surely agreed

with those in initially generated HDAC2(-/-) mutants mentioned above [Chap. 2]. 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, and represented two different ranges of vision within microscopy (Fig. 4-5). Interestingly, these mutant clones were morphologically observed to be aggregative form at the early (E) stage but dispersive form, like the form of DT40 cells and also that of Pax5(-) mutants, at the late (L) stage [Chap. 3].

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 4-3A and 4-3B, the 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. 4-1C, 4-1D and 4-2), were altered in the six HDAC2(-/-) mutant clones during cultivation. We examined changing patterns more minutely in gene expressions of IgM H- and L-chains and also those of altered HATs, HDACs and transcription factors (and Ikaros and XBP-1) in four mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6, but not in two remaining clones cl.2-3 and cl.2-5 during cultivation. Because, the latter two clones had great resemblance to clones cl.2-2 and cl.2-4 in many cellular properties as mentioned above. 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 some in-between 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 β-actin as control (Fig. 4-6). The levels of whole and secreted forms of IgM H-chain mRNA were dramatically elevated at the early (E) stage in all of four mutant clones. Thereafter, the elevated levels of these two type mRNAs were gradually/dramatically decreased via 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 four mutant clones the mRNA level of IgM L-chain was slightly elevated at the early (E) stage and slowly reduced 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. 4-6). Remarkably, the changing patterns in gene expressions of PCAF, HDAC7 and HDAC9 were clearly distinct in each of individual clones of HDAC2(-/-) mutants during cultivation as follows. The mRNA level of PCAF, which was a very low in

DT40 cells, was dramatically elevated until the in-between stages (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 four mutant clones during cultivation. That is, the mRNA level of HDAC7 in these clones was obviously decreased at the early (E) stage but certainly increased until the in-between stages (17 ~ 27 days) and thereafter remained unchanged or very slowly decreased. Moreover, the mRNA level of HDAC7 at any cultivation stages in these four 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 in these four mutant clones up to the in-between stage (22 days), and thereafter the elevated 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, PU.1 and XBP-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. 4-6). Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, XBP-1 and OBF1 (but not Blimp1) genes were sufficiently or considerably expressed in DT40 cells. Surprisingly, however, gene expression patterns of these transcription factors (except XBP-1) were complicate/diverse in 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 the 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, the mRNA levels of Pax5 and Aiolos were almost completely decreased at the early (E) stage, and thereafter remained unchanged or were very slightly increased during cultivation until the late (L) stage. Remarkably, in three residual clones (cl.2-2, cl.2-4 and cl.2-6), the mRNA levels of Pax5 and Aiolos were completely decreased at the early (E) stage, but thereafter these reduced mRNA levels were gradually/surely increased during cultivation. Namely, at the late (L) stage, those of Pax5 and Aiolos were approximately 20 ~ 40-folds or 80 ~ 120-folds of the levels 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, the 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 as a whole but a remarkably different changing pattern in the latter one 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/certainly 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 elevated at the late (L) stage to about 60 ~ 100% of that in DT40 cells. By contrast, in clone cl.2-1, that of OBF1 was gradually/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 decreased until the late (L) stage to about 50% of this initial 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 mutant 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 four mutant clones to about 200 ~ 300% of that in DT40 cells and thereafter slowly decreased 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 four mutant clones to about 30 ~ 50% of that in DT40 cells, and thereafter slightly increased until the late (L) stage in clone cl.2-1 or 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 four mutant clones during cultivation.

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

As a typical cellular property, we finally studied growth rate of individual HDAC2(-/-) mutant clones (Fig. 4-7). 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 the 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 its generation (birth) by gene targeting techniques (Figs. 4-1, 4-2 and 4-4) because HDAC2, as a supervisor, regulates transcriptions of these two immunoglobulin genes through opposite control of Pax5, Aiolos, EBF1, OBF1, and also Ikaros plus E2A gene expressions in wild type DT40 cells [2, 7, Chap. 2]. These results in DT40 and HDAC2(-/-) mutant cells at the early cultivation stage are schematically shown in Figures 4-8 and 4-9. The majority of artificially accumulated IgM H- and L-chains in HDAC2(-/-) exist as a native soluble form capable of building a high molecular weight complex with each other probably within endoplasmic reticulum [2]. 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(-/-) [1, 2]. In addition, HDAC2(-/-) mutant cells exist rather as a morphologically aggregative (and also probably distorted) form at the early stage (Figs. 4-4 and 4-5), 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, artificially accumulated protein levels of IgM H- and L-chains at the early stage in HDAC2(-/-) are gradually reduced during cultivation and at the late stage reached to comparable levels in DT40 cells (Figs. 4-2 and 4-4). In parallel with these changes, morphology of HDAC2(-/-) also changes; i.e., the aggregative form at the early stage is altered during cultivation to the dispersive form, like that of DT40 cells, at the late stage and it must be comfortable (or peaceful) for both of DT40 and HDAC2(-/-) mutant cells (Fig. 4-5). Moreover, the morphology of HDAC2(-/-) at the early stage and its changing patterns during cultivation are clearly different from those of Pax5(-) [Chap. 3].

Interestingly, as clearly presented in Figure 4-6, mRNA levels of various altered transcription factors (and chromatin modifying enzymes) showed following distinct changing patterns during cultivation among six individual clones of HDAC2(-/-) mutants tested, in spite of almost similar changing pattern in protein and mRNA levels of IgM H- and L-chains (Figs. 4-1, 4-2, 4-3 and 4-6) and also in cell morphology (Figs. 4-4 and 4-5). That is, remarkably, in clone cl.2-1 the mRNA levels of Pax5, Aiolos and EBF1, all of which down-regulate transcriptions of IgM H- and L-chain genes [2, 7, Chaps. 2 and 3], are dramatically reduced at the early stage and remained unchanged until the late stage during cultivation. On the other hand, the mRNA level of OBF1, which probably up-regulates transcriptions of these two immunoglobulin genes [19], is gradually/dramatically decreased until the late stage. In clones cl.2-2 and cl.2-4 (and also cl.2-3 plus cl.2-5) the mRNA levels of Pax5, Aiolos and EBF1 are dramatically reduced at the early stage, and thereafter mRNA levels of Pax5 and Aiolos are gradually elevated until the late stage during cultivation but very low mRNA level of EBF1 remained unchanged. In contrast, the mRNA level of OBF1 insignificantly changes during cultivation. In clone cl.2-6 the 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 we collected the mutant cells at ~10 to 12 days after their birth by gene targeting techniques [Chaps. 2, 3 and 4] and their doubling times were ~12 hrs [1, 2, 8]; therefore, they should be populations of ~20 to 25 generations even at the early stage.

In summary, clone cl.2-1 at the late cultivation stage seems to be OBF1-dependent type and distinct from wild type DT40 cells in appearance in manner for gene expressions of IgM H- and L-chains. Clones cl.2-2, cl.2-3, cl.2-4 and cl.2-5 at the late stage seem to be Pax5- and Aiolos-dependent type and slightly similar to DT40 cells in appearance in manner for gene expressions of the two immunoglobulin proteins. These four mutant clones should be major type, since four initially generated HDAC2(-/-) mutant clones roughly resembled them in several characteristics [1, 2]. Clone cl.2-6 at the late stage seems to be Pax5-, Aiolos- and EBF1-dependent type and most similar to DT40 cells in appearance in manner for the two immunoglobulin gene expressions. These results in individual clones of HDAC2(-/-) mutants at the late cultivation stage are schematically shown in Figure 4-10. On the other hand, alterations in the mRNA level of E2A, which up-regulates transcriptions of IgM H- and L-chain genes [2], must be such unrelated to the decreases in the two immunoglobulin protein levels in HDAC2(-/-) during cultivation, since its alteration was not remarkable in all of the six mutant clones (Figs. 4-3 and 4-6). Further, 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 [2], and the IgM H-chain (but not L-chain) gene expression was slightly down-regulated by GCN5-deficiency [20]. Anyhow, the above-mentioned speculation on classification of HDAC2(-/-) mutant clones 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 different from that of DT40 cells (Fig. 4-7). If additional independent HDAC2(-/-) mutant clones are analyzed, as manners for gene expressions of IgM H- and L-chains, besides the above-mentioned three types, other distinct types will be probably added. Moreover, such differences in gene expressions of numerous transcription factors and chromatin modifying enzymes among the examined individual mutant clones also suggest 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. Besides, distinct changing patterns of gene expressions of various transcription factors and cell morphology as mentioned above, the decreasing speeds of protein and mRNA levels of IgM H- and L-chains during cultivation were obviously different between HDAC2(-/-) and Pax5(-) mutants [Chap. 3]. These results suggest that the

above-mentioned three manners (and supposed ones) to decrease gene expressions of IgM H- and L-chains in HDAC2(-/-) mutant cells should be certainly different from those in Pax5(-) mutant cells.

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 during cultivation. Namely, the same and new cell functions mean not only excluding artificially accumulated IgM H- and L-chains which are uncomfortable for the mutant cells themselves, but also ridding themselves free from aggregative form. These distinct manners should be definitely based on diverse changes in gene expressions of chromatin modifying enzymes and transcription factors (such as PCAF, HDAC7, HDAC9, Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, Blimp1, OBF1 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, since the changing patterns in gene expressions of the four factors were in anti-parallel or parallel with those in transcriptions of the two immunoglobulin genes in one or more of individual HDAC2(-/-) mutant clones. Based on these results, for manners to eliminate artificially accumulated IgM H- and L-chains as uncomfortable intra-cellular environment change (and/or extra-cellular one, if existence) in HDAC2(-/-) mutants, we propose a brief working hypothesis as follows (Fig. 4-11).

The accumulation of IgM H- and L-chains in HDAC2(-/-) mutants should be recognized as uncomfortable environment change and 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. The environment change should induce alterations in chromatin structure of a set of various chromatin modifying enzyme and transcription factor genes (PCAF, HDAC7, HDAC9, Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, OBF1, Blimp1, XBP-1, Oct2, etc.), resulting in their altered transcription levels. The response(s) for the environment change should be successively converged to chromatin structure of several particular factor and enzyme genes (Pax5, Aiolos, EBF1, OBF1, PCAF, HDAC7, HDAC9, etc.) in individual mutant clones via a lot of generations during cultivation, and thereby inducing 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 function to exclude accumulated IgM H- and L-chain proteins based on their decreased gene expressions in different manners through varied gene expressions of particular transcription factors (Pax5, Aiolos, EBF1, OBF1, etc.). Therefore, concerning exclusion of artificially accumulated IgM H- and L-chains, one of the most interesting subjects in this working hypothesis is elucidation of distinct manners for gene expressions of these particular transcription factors (and also chromatin modifying enzymes) in individual clones of HDAC2(-/-) mutants via a lot of generations (cell divisions) during continuous cultivation.

METHODS

Cell cultures

HDAC2(-/-) mutant clones were continuously cultivated as described [1, 2, Chaps. 2 and 3] and collected at indicated interval periods, including the early (E), middle (M) and late (L) stages of cultivation. These cultivation periods and/or stages were practically counted from the first stock day (see latter). DT40 (W) was used as control. Cell numbers were counted at indicated times to determine growth rate as described [1, 2, Chap. 3].

Generation of HDAC2-deficient DT40 mutant cell line 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 [1], using appropriate restriction enzymes (SpeI plus SphI, and SphI plus BamHI), and transferred into pBluescript II vector as described [1, 2]. The 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 arms. In the resultant targeting vectors, therefore, genomic nucleotide sequences corresponding to exons 6 to 7 of the gene were replaced with the drug resistance cassettes, since the 3.5-kb and 2.3-kb fragments fully corresponded to the parts of the introns 2 to 5 and intron 7, respectively (Fig. 1A). Transfection was carried out as described [1, 2, 8, 16]. To obtain HDAC2-deficient 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 the transfectant, in which one of two HDAC2 alleles had already been disrupted, and selected stable transfectants in medium containing 400 μg of Zeocine and 2 mg of hygromycin 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 [1, 2, 21, Chap. 3]. 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(-/-) 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 Supplementary Table 4-S1 and our previous papers [2, 22, Chap. 2] as described [2, Chaps. 2 and 3]. β-actin was used as control. 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 [2, 22]. 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 are indicated as percentages of control values obtained from DT40 cells (data not shown).

Western blotting

Western blotting was performed as described [1, 2, Chaps. 2 and 3]. 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) stages and DT40 cells (W) (5×10^6 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 anti-serum 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). Data analyses were carried out by Multi Gauge Ver3.X software using a luminescent image analyzer LAS-1000plus (FUJIFILM). Relative amounts of IgM H-chain and large (high) plus small (low) forms of IgM L-chain were measured (data not shown).

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 [3, 4, 23, Chap. 3]. Post-embedding immunocytochemistry using immunogold labeling was carried out as described [24, Chaps. 2 and 3]. 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 (W) as control, 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 a control, primary antibody was omitted or replaced by normal goat serum.

REFERENCES

[1] Takami, Y., Kikuchi, H. and Nakayama, T.: Chicken histone deacetylase-2 controls the amount of the IgM H-chain at the steps of both transcription of its gene and alternative processing of its pre-mRNA in the DT40 cell line. J. Biol. Chem. 274, 23977-23990, 1999.

[2] Nakayama, M., Suzuki, H., Yamamoto-Nagamatsu, N., Barman, H. K., Kikuchi, H., Takami, Y., Toyonaga, K., Yamashita, K. and Nakayama, T.: HDAC2 controls IgM H and L-chain gene expressions via EBF1, Pax5, Ikaros, Aiolos and E2A gene expressions. Genes Cells 12, 359-373, 2007.

[3] Toyonaga, K., Kikuchi, H., Yamashita, K., Nakayama, M., Chijiiwa, K. and Nakayama, T.: E2A participates in a fine control of pre-mature B cell apoptosis mediated by B cell receptor signaling via transcription regulations of survivin, IAP2 and caspase-8 genes. FEBS J. 276/5, 1418-1428, 2009.

[4] Kikuchi, H., Yamashita, K., Nakayama, M., Toyonaga, K., Tsuneyoshi, I., Takasaki, M. and Nakayama, T.: Lacking of Aiolos accelerates pre-mature B cell apoptosis mediated by BCR signaling through elevation in cytochrome c release. BBA-Molecular Cell Research 1793, 1304-1314, 2009.

[5] Kikuchi, H., Nakayama, M., Takami, Y., Kuribayashi, F. and Nakayama, T.: Possible involvement of Helios in controlling the immature B cell functions via transcriptional regulation of protein kinase Cs. Results Immunol. 1, 88-94, 2011.

[6] Kikuchi, H., Nakayama, M., Takami, Y., Kuribayashi, F. and Nakayama, T.: EBF1 acts as a powerful repressor of Blimp-1 gene expression in immature B cells. Biochem. Biophys. Res. Commun. 422, 780-785, 2012.

[7] Kikuchi, H., Barman, H. K., Nakayama, M., Takami, Y. and Nakayama, T.: Studies on epigenetic control of B cell functions using the DT40 cell line. Advances in Genetics Research 2, Urbano K. V. (Ed.), Nova Science Publishers, Inc. NY, pp153-166, 2010.

[8] Takami, Y., Takeda, S. and Nakayama, T.: Targeted disruption of an H3-IV/H3-V gene pair causes increased expression of the remaining H3 genes in the chicken DT40 cell line. J. Mol. Biol. 250, 420-433, 1995.

[9] Seguchi, K., Takami, Y. and Nakayama, T.: Targeted disruption of 01H1 encoding a particular H1 histone variant causes changes in protein patterns in the DT40 chicken B cell line. J. Mol. Biol. 254, 869-880, 1995.

[10] Takami, Y., Takeda, S. and Nakayama, T.: Targeted disruption of H2B-V encoding a particular H2B histone variant causes changes in protein patterns on two-dimensional polyacrylamide gel electrophoresis in the DT40 chicken B cell line. J. Biol. Chem. 270, 30664-30670, 1995.

[11] Takami, Y., Takeda, S. and Nakayama, T.: An approximately half set of histone genes is enough for cell proliferation and a lack of several histone variants causes protein pattern changes in the DT40 chicken B cell line. J. Mol. Biol. 265, 394-408, 1997.

[12] Takami, Y. and Nakayama, T.: One allele of the major histone gene cluster is enough for cell proliferation of the DT40 chicken B cell line. Biochim. Biophys. Acta 1354, 105-115, 1997.

[13] Takami, Y. and Nakayama, T.: A single copy of linker H1 genes is enough for proliferation of the DT40 chicken B cell line, and linker H1 variants participate in regulation of gene expression. Genes Cells 2, 711-723, 1997.

[14] Sanematsu, F., Takami, Y., Barman, H. K., Fukagawa, T., Ono, T., Shibahara, K. and Nakayama, T.: Asf1 is required for viability and chromatin assembly during DNA replication in vertebrate cells. J. Biol. Chem. 281, 13817-13827, 2006.

[15] Takami, Y., Ono, T., Fukagawa, T., Shibahara, K. and Nakayama, T.: Essential role of CAF-1-mediated rapid nucleosome assembly for DNA replication and cell division in vertebrate cells. Mol. Biol. Cell 18, 129-141, 2007.

[16] Baba, T. W., Giroir, B. P. and Humphries, E. H.: Cell lines derived from avian lymphomas exhibit two distinct phenotypes. Virology 144, 139-151, 1985.

[17] Buerstedde, J.-M. and Takeda, S.: Increased ratio of targeted to random integration after transfection of chicken B cell lines. Cell 67, 179-188, 1991.

[18] Takeda, S., Masteller, E. L., Thompson, C. B. and Buerstedde, J.-M.: RAG-2 expression is not essential for chicken immunoglobulin gene conversion. Proc. Natl. Acad. Sci. USA 89, 4023-4027, 1992.

[19] Takechi, S., Adachi, M. and Nakayama, T.: Cloning and characterization of the chick Oct binding factor OBF-1. Biochim. Biophysica Acta 1577, 466-470, 2002.

[20] Kikuchi, H., Nakayama, M., Kuribayashi, F., Imajoh-Ohmi, S., Nishitoh, H., Takami, Y. and Nakayama T.: GCN5 is involved in regulation of immunoglobulin heavy chain gene expression in immature B cells. Gene 544, 19-24, 2014.

[21] Southern, E. M.: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517, 1975.

[22] Kikuchi, H., Takami, Y. and Nakayama, T.: GCN5: a supervisor in all-inclusive control of vertebrate cell cycle progression through transcription regulation of various cell cycle-related genes. Gene 347, 83-97, 2005.

[23] Kikuchi, H. and Nakayama, T.: GCN5 and BCR signaling collaborate to induce pre-mature B cell apoptosis through depletion of ICAD and IAP2 and activation of caspase activities. Gene 419, 48-55, 2008.

[24] Bendayan, M. and Zollinger, M.: Ultrastructural localization of antigenic sites on osmium-fixed tissues applying the protein A-gold technique. J. Histochem. Cytochem. 31, 101-109, 1983.

FIGURE LEGENDS

Figure 4-1. Genomic organization of chicken HDAC2 gene, generation of HDAC2(-/-) DT40 mutant cells and alterations in protein and mRNA levels of IgM H- and L-chains in HDAC2(-/-) DT40 mutant clones during cultivation

A. Schematic representation of chicken HDAC2 genomic locus (top) with enlarged drawing of the targeted region (middle) and its 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). The 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 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 using equal amounts of total RNAs and appropriate primers for HDAC2 (HDAC2), and 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). Chicken β-actin was used as control.

Figure 4-2. Alterations in amounts of IgM H- and L-chains in HDAC2(-/-) DT40 mutant clones 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 Fig. 4-1. The protein bands were cut off and represented separately. Upper and lower bands indicated by IgM H, respectively, correspond to those of IgM H-chain detected by antibodies for IgM H- and L-chains. IgM L-chain (indicated by IgM L) was detected as two bands.

Figure 4-3. Alterations in gene expressions of HDACs, HATs and transcription factors in HDAC2(-/-) DT40 mutant clones during cultivation

Total RNAs used were the same ones as in Fig. 4-1D. RT-PCR was performed in the same manner, using appropriate primers for members of HDACs and HATs (A) and transcription factors (B), instead of primers used in Fig. 4-1D.

Figure 4-4. Alterations in amounts of IgM H-chain in HDAC2(-/-) DT40 mutant clones 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 were observed only at the early (E) cultivation stage in four mutant clones (E in upper panels). Positive signals for IgM H-chain proteins were also observed only at the early (E) cultivation stage in four mutant clones (E in lower panels).

Figure 4-5. Alterations of morphology of HDAC2(-/-) DT40 mutant clones 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 distinct ranges of vision. Aggregative form was observed only at the early (E) stage in four mutant clones.

Figure 4-6. Alterations in gene expressions of IgM H- and L-chains, HATs, HDACs and transcription factors in HDAC2(-/-) DT40 mutant clones during cultivation

Total RNAs were extracted from 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 in the same manner, using appropriate primers for HDAC2, IgM H- and L-chains, and members of HATs, HDACs and transcription factors, which were observed to alter in Figs. 4-1D and 4-3.

Figure 4-7. Growth rates of HDAC2(-/-) DT40 mutant clones

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. 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 4-8. 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 4-9. Models for roles of Pax5, Aiolos and EBF1 in control of gene expressions of IgM H- and L-chains at early stage of cultivation in all clones of HDAC2(-/-) DT40 mutants

Figure 4-10. Models for roles of Pax5, Aiolos, EBF1 and OBF1 in control of gene expressions of IgM H- and L-chains at late stage of cultivation in individual clones of HDAC2(-/-) DT40 mutants

Figure 4-11. A model for manners to exclude IgM H- and L-chains accumulated in HDAC2(-/-) DT40 mutant cells during cultivation