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Review

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

Masami Nakayama[1] and Tatsuo Nakayama[1,2,*]

[1]Section of Biochemistry and Molecular Biology, Department of Medical Sciences, Faculty of Medicine; [2]Department of Life Science, Frontier Science Research Center, University of Miyazaki, 5200, Kihara, Kiyotake, Miyazaki, 889-1692, Japan.

ABSTRACT

We recently generated histone deacetylase2 (HDAC2)-deficient DT40 mutants HDAC2(-/-) and analyzed their characteristics at various intervals during cultivation. In this article we reviewed our studies on several characteristics of HDAC2(-/-) mutants. In six independent clones (cl.2-1 to cl.2-6) of HDAC2(-/-) mutants, proteins and mRNAs of IgM H- and L-chains were dramatically increased at the early stage of cultivation, and thereafter decreased in almost similar changing pattern and at the 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 six individual HDAC2(-/-) clones; 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. These results revealed that individual clones of HDAC2(-/-) mutants have the ability to gain new cell function to exclude artificially accumulated IgM H- and L-chains depending on alterations in gene expressions of Pax5, Aiolos, EBF1, OBF1, etc. 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

*Corresponding author
tnakayam@med.miyazaki-u.ac.jp

has been undoubtedly thought to be one of the most fundamental and important ways of expression of cell functions in eukaryotes. For the last several decades, mechanisms to modulate chromatin structure with epigenetic modifications, such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, etc., 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 catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs) as chromatin-modifying enzymes are surely major ones [2-14]. Even in the last few years, countless numbers of researches on such epigenetic modifications of chromatin have been vigorously continued without interruptions in more diverse life science fields, e.g., gene expression/transcription, DNA replication, differentiation, development, memory, pluripotency, clinical medicine and so on [15-36]. Using gene targeting techniques in the chicken B cell line DT40 possessing homologous recombination with a very high frequency [37-39], we have systematically studied *in vivo* roles of a large number of specific members of HATs, HDACs and transcription factors [40-47], besides histones and histone chaperones [48-55].

Our analyses of initially generated HDAC2-deficient DT40 mutants HDAC2(-/-) showed that HDAC2 controls the amount of IgM H-chain through the regulation of both its gene expression and alternative pre-mRNA processing [40]. Moreover, we revealed that the HDAC2 deficiency increases gene expressions of HDAC4, HDAC9, PCAF plus E2A as well as IgM H- and L-chains, decreases those of HDAC7, Pax5, Aiolos, Ikaros plus EBF1, and changes bulk acetylation levels of several particular Lys residues (K) of core histones H2A, H2B, H3 and H4 [41]. Therefore, to know the 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 existing on Z sex chromosome [47] and Ikaros-down mutant cell line Ikaros(-/-/+) devoid of two of three Ikaros alleles existing on chromosome 2 (our unpublished data). These results were consistent with the previous findings that the DT40 cell line has 38 autosomal chromosome pairs, with the exception of chromosome 2, which is triploid, and the Z/W sex chromosomes [56, 57]. 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].

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 the following remarkable phenomena [42]. In [42], the cultivation stages and/or periods were practically counted from the first day of cultivation from the stock at -80 °C. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) showed that in HDAC2(-/-) mutants IgM H- and L-chains were dramatically elevated at the early stage of cultivation and thereafter gradually reduced during cultivation and at the later stage reached comparable levels as in DT40 cells, whereas changes in cellular levels of most of other major proteins were insignificant during cultivation. Western blotting carried out at shorter intervals, using antibody specific for chicken IgM L-chain that cross-reacts with IgM H-chain, sufficiently confirmed the above-mentioned results obtained by 2D-PAGE. Immuno-electron microscopy using antibody specific for IgM H-chain also showed that the immunoglobulin proteins were 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 were dramatically increased at the early stage, and thereafter gradually decreased during cultivation and at the later stage reached a level very close to that in DT40 cells. These results, together, indicated that in HDAC2(-/-) mutants IgM H- and L-chains were dramatically

and considerably accumulated depending on their increased gene expressions at the early stage, gradually reduced depending on their decreased gene expressions during cultivation, and at the later stage finally reached 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 were separately altered 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 were gradually increased during cultivation. Furthermore, we cloned and sequenced ~4.9 kb 5'-upstream region of the Pax5 gene from DT40 genomic DNA by our original gene walking techniques (GenBank accession no.: LC060666). Interestingly, qualitative chromatin immuno-precipitation (ChIP) assay suggested that acetylation levels of Lys-9 residue of histone H3 (K9/H3) within some segments of chromatin structure surrounding proximal ~2.0 kb 5'-upstream region of the Pax5 gene were decreased at the early stage in HDAC2(-/-) mutants and at the later stage increased and reached comparable levels as in DT40 cells. Of the newly obtained results from the initially generated HDAC2(-/-) mutants, it should be worth of special mention that artificially and excessively accumulated IgM H- and L-chains at the early cultivation stage were diminished depending on their decreased gene expressions, which is mainly attributed to altered gene expressions of the above-mentioned specific transcription factors during cultivation [42]. Besides, in Pax5(-) mutants these two immunoglobulin proteins, artificially and dramatically accumulated at the early stage, were more rapidly reduced depending on their decreased gene expressions, and this is probably attributed to altered gene expressions of some of specific transcription factors and chromatin-modifying enzymes during cultivation [47].

In order to closely clarify the mechanisms that cause decreases in gene expressions of IgM H- and L-chains during cultivation, in [58] we generated again homozygous HDAC2-deficient DT40 mutants HDAC2(-/-) by gene targeting techniques [37-39], because these techniques were very powerful for studying physiological roles of numerous genes of HATs, HDACs, transcription factors, histones and histone chaperones as mentioned above [40-55]. Analyses of six individual clones (cl.2-1 to cl.2-6) of HDAC2(-/-) mutants revealed that 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 a model for the role of HDAC2 in diminishing IgM H- and L-chains accumulated excessively in individual clones of HDAC2(-/-) mutants during continuous cultivation [58]. This article is the review of our studies in ref. 58.

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

As shown in fig. 1A, genomic DNA of the chicken HDAC2 consists of 16 exons, different from 14 exons reported previously [40]. We newly generated homozygous HDAC2-deficient DT40 mutant cells HDAC2(-/-) devoid of two HDAC2 alleles by gene targeting techniques using two targeting vectors containing MerCreMer/bleo and hyg [58], essentially as described in [37-41, 48-55]. 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. 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 fig. 1B. In all of these six clones, endogenous ~4.0 kb band derived from intact alleles disappeared and ~7.0 kb band derived from two targeted alleles newly appeared. Residual 22 clones also showed the same results. RT-PCR using primers specific for HDAC2 showed no band for mRNA originated from the intact HDAC2 gene in the six clones (Fig. 1D), confirming that these six clones (and residual 22 clones) are homozygous HDAC2-deficient DT40 mutant cells HDAC2(-/-).

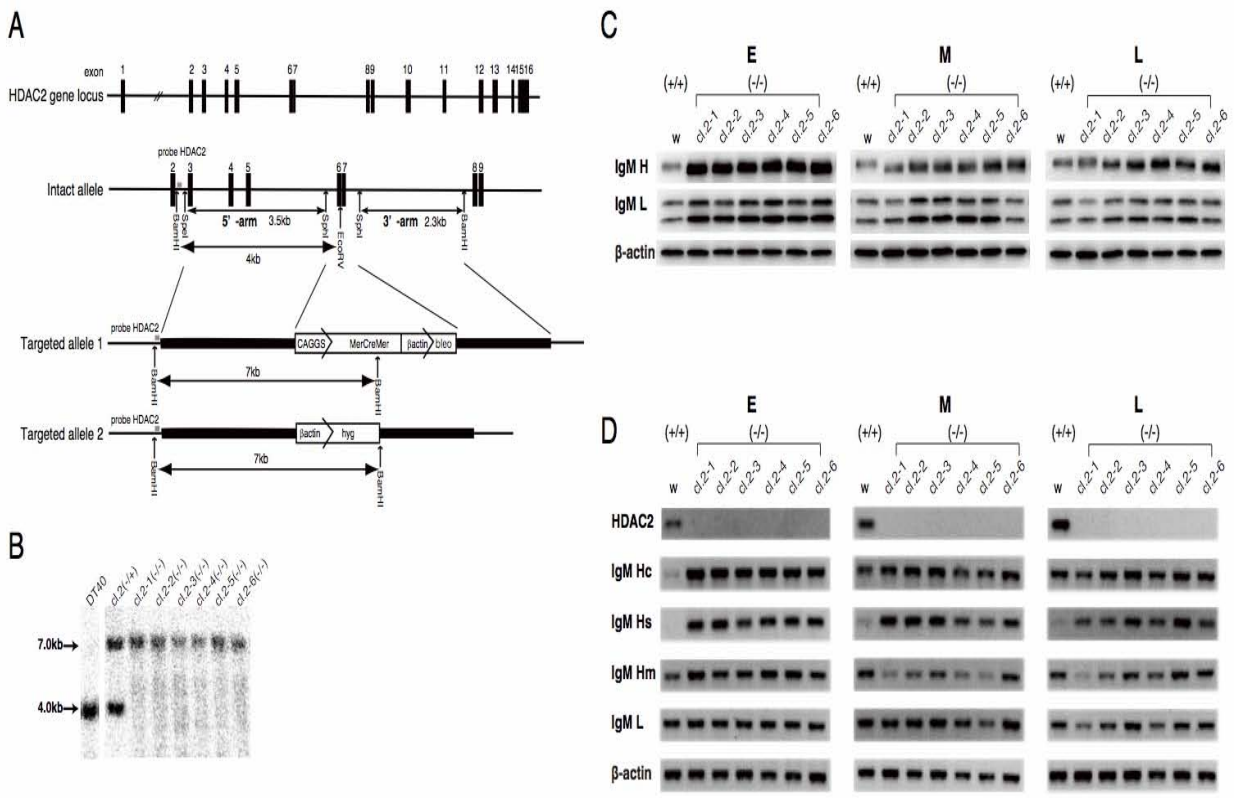

Fig. 1. Genomic organization of chicken HDAC2 gene, generation of HDAC2-deficient DT40 mutants and alterations in protein and mRNA levels of IgM H- and L-chains in HDAC2(-/-) DT40 mutant clones during continuous cultivation.

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. Possible relevant fragments obtained from BamHI and EcoRV digestions are shown with their lengths in kb. **B.** Southern blotting of homologous recombination. 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 proteins were prepared from DT40 cells (W) and 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), middle (M) and later (L) cultivation stages, and analyzed by Western blotting, using anti-chicken IgM L-chain and H-chain antibodies. IgM H and IgM L indicate IgM H-chain and IgM L-chain (as two bands), respectively. **D.** RT-PCR. Total RNAs were extracted from DT40 cells (W) and 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), middle (M) and later (L) cultivation stages, and analyzed by RT-PCR, using appropriate primers for whole IgM H-chain mRNA (IgM Hc), its secreted form (IgM Hs), its membrane-bound form (IgM Hm) and IgM L-chain mRNA (IgM L). The figure is identical with fig. 1 of ref. [58].

Protein and mRNA levels of IgM H- and L-chains in HDAC2(-/-) DT40 mutants change during continuous cultivation

First, 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 later (L; 53 days) stages of cultivation, and from DT40 cells (W; wild-type) (Fig. 1C). 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 drastically decreased in all of these mutant clones during cultivation. The protein

level of IgM L-chain (detected as two bands) at the early (E) stage in all of these HDAC2(-/-) mutant clones was considerably increased compared with that in DT40 cells, and thereafter gradually decreased during cultivation and at the later stage reached a level very close to that in DT40 cells.

Next, 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 collected at the early (E), middle (M) and later (L) stages, and from DT40 cells (W) (Fig. 1D). As described previously [40-42, 47], primers IgM Hc, IgM Hs plus IgM Hm and IgM L could specifically detect whole, secreted plus membrane-bound forms of IgM H-chain mRNA and IgM L-chain mRNA, respectively. 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 types of mRNAs in all of the mutant clones were considerably decreased at the middle (M) stage and dramatically decreased at the later stage and reached a level very close to that 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 changed slightly or remained unchanged at any cultivation stages.

Increased protein levels of IgM H- and L-chains caused by HDAC2-deficiency are dramatically and gradually decreased in individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation

The above-mentioned result that protein and mRNA levels of IgM H- and L-chains in HDAC2(-/-) mutants dramatically changed during cultivation seems to be a very important phenomenon. To confirm these findings, we carried out Western blotting more minutely at shorter intervals, using two antibodies specific for chicken IgM H-chain and considerably specific for chicken IgM L-chain that cross-reacts with IgM H-chain. Total cellular proteins were prepared from six HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6), cultivated for various indicated periods including the early (E), middle (M) and later (L) stages, and from DT40 cells (W) (Fig. 2). 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 drastically decreased through 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, 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 through 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 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 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 various genes encoding respective members of HDACs and HATs, on total RNAs prepared from six HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6), which were collected at the early (E), middle (M) and later (L) stages, and from DT40 cells (W) (Fig. 3A). In the six mutant clones, mRNA levels of HDAC7, HDAC9 and PCAF

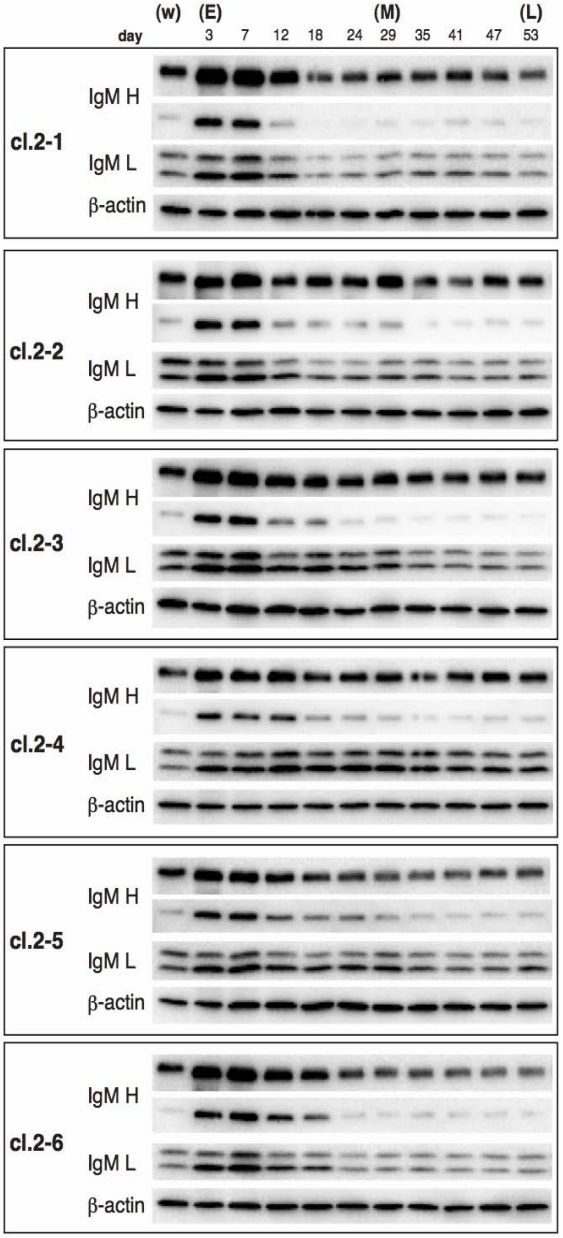

Fig. 2. Alterations in amounts of IgM H- and L-chains in individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation.

Western blotting was performed on total cellular proteins prepared from DT40 cells (W) and HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) at the indicated cultivation periods, including the early (E), middle (M) and later (L) cultivation stages. Upper and lower bands (indicated by IgM H) correspond to IgM H-chain detected by antibodies for IgM H- and L-chains, respectively. IgM L-chain (two bands indicated by IgM L) was detected by antibody for IgM L-chain. The figure is identical with fig. 2 of ref. [58].

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. Detailed changing patterns in their gene expressions in four mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) will be shown later.

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

Immuno-electron microscopy using antibody specific for chicken IgM H-chain showed that in initially generated HDAC2(-/-) mutant cells [40] the immunoglobulin proteins were clearly accumulated at the early (E) stage and thereafter obviously reduced at the later (L) stage and reached almost the same level as in DT40 cells (W) [42]. To explore whether newly generated HDAC2(-/-) mutant cells morphologically change during cultivation, we first examined the morphology of four mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) and DT40 cells. Electron microscopy showed

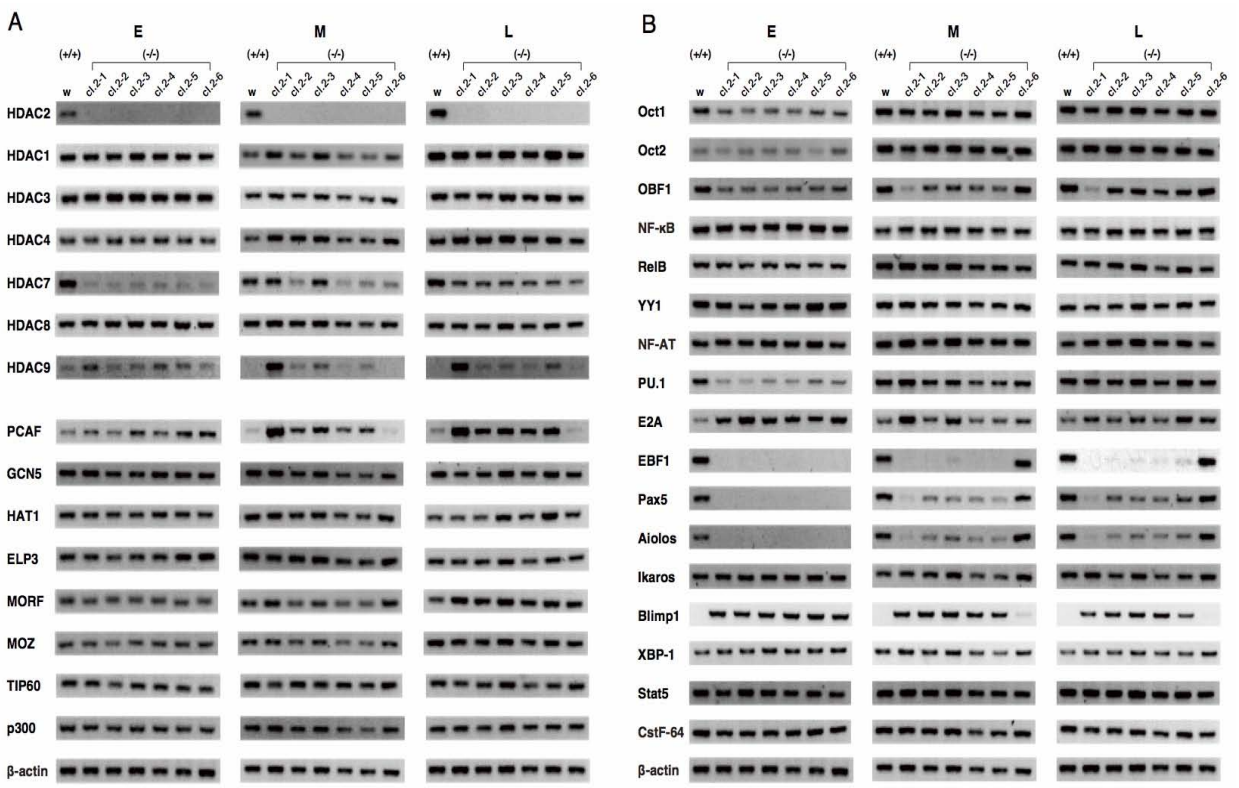

Fig. 3. Alterations in gene expressions of specific members of HDACs, HATs and transcription factors in individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation.

RT-PCR was performed on total RNAs identical with those shown in fig. 1D, using appropriate primers for various members of HDACs and HATs (A) and transcription factors (B). The figure is identical with fig. 3 of ref. [58].

that all of these mutant clones were 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 [42, 58]. 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 the mutant clones, and thereafter most of the accumulated proteins disappeared at the later (L) stage, like in DT40 cells [42, 58]. 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 [42, 58]. Interestingly, these 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, 58].

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 figs. 3A and 3B, mRNA levels of PCAF, HDAC7, HDAC9, Pax5, Aiolos, EBF1, E2A, PU.1, Blimp1 and OBF1, as well as protein and mRNA levels of IgM H- and L-chains (Figs. 1C, 1D and 2), were altered in the six tested HDAC2(-/-) mutant clones during cultivation. We very closely examined the changing patterns in gene expressions of IgM H- and L-chains and altered members of HATs, HDACs and transcription factors (and 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 mutant clones collected at the indicated periods; i.e., at the early (E) stage, several in-between cultivation periods including middle (M) stage, and 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 plus IgM L, and HDAC2 (Fig. 4). The levels of whole and secreted forms of IgM H-chain mRNA were dramatically increased at the early (E) stage in all of the four mutant clones, and thereafter 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 as in DT40 cells. The increased level of membrane-bound form of IgM H-chain mRNA at the early (E) stage was 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

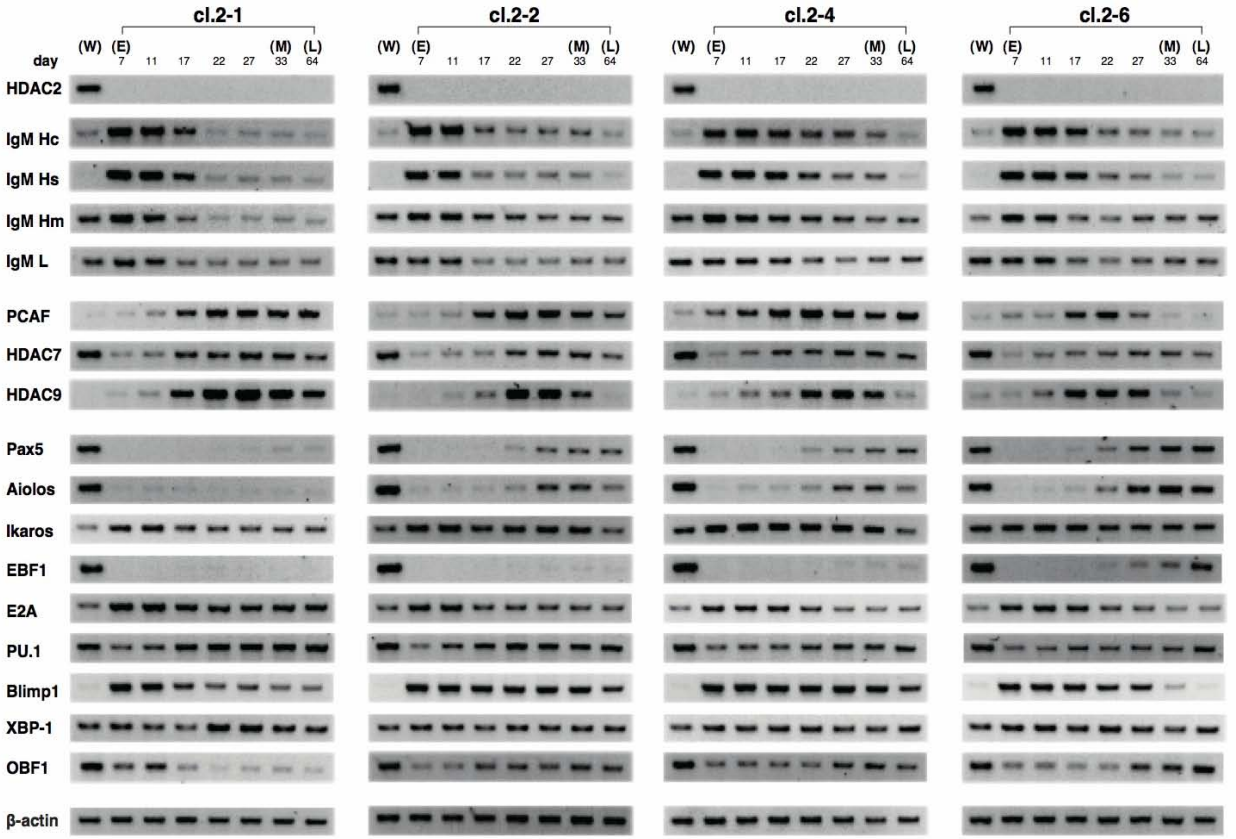

Fig. 4. Alterations in gene expressions of IgM H- and L-chains, specific members of HATs, HDACs and transcription factors in individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation.

Total RNAs were extracted from DT40 cells (W) and four HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) at the indicated cultivation periods, including the early (E), middle (M) and later (L) cultivation stages. RT-PCR was performed on total RNAs, using appropriate primers for IgM H- and L-chains, and specific members of HATs, HDACs and transcription factors, most of which were altered in figs. 1D and 3. The figure is identical with fig. 6 of ref. [58].

above-mentioned altered chromatin-modifying enzyme genes (Fig. 4). Remarkably, the changing patterns in gene expressions of PCAF, HDAC7 and HDAC9 were clearly distinct in individual clones of HDAC2(-/-) mutants during cultivation as follows. The mRNA level of PCAF, which was 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 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 these 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 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 change dramatically or moderately 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 altered transcription factor genes (Fig. 4). 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 the 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. 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 increased dramatically 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 patterns 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, 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 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 continuous cultivation is different from each other

As a typical cellular property, we studied growth rate of individual HDAC2(-/-) mutant clones at the later (L) cultivation stage (Fig. 5). 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.

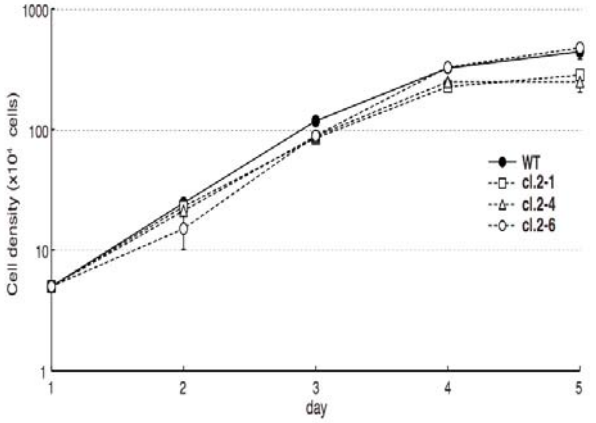

Fig. 5. Growth rate of individual clones of HDAC2(-/-) DT40 mutants.

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 the indicated times. The numbers are plotted on a log phase. Symbols for the clones are shown in the figure. The figure is identical with fig. 7 of ref. [58].

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) (Figs. 1 and 2), because HDAC2 as a supervisor indirectly regulates the gene expressions of the two immunoglobulin proteins through opposite control of Pax5, Aiolos, EBF1, OBF1, E2A and Ikaros gene expressions in wild-type DT40 cells [41, 42]. These results obtained from DT40 and HDAC2(-/-) mutant cells at the early cultivation stage are schematically shown in fig. 6. The majority of IgM H- and L-chains accumulated in HDAC2(-/-) mutants exist as a native soluble form capable of building a high-molecular weight complex with each other probably within the 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 form at the early stage [58], the real reason for which is still unknown. Anyhow, the accumulated immunoglobulin proteins and the aggregative form should be abnormal environments to HDAC2(-/-) mutant cells. Surprisingly, the elevated protein levels of IgM H- and L-chains accumulated at the early stage in HDAC2(-/-) mutants are dramatically reduced during cultivation and at the later stage reached comparable levels as in DT40 cells (Fig. 2). In parallel with the change, morphology of HDAC2(-/-) mutant cells also changes; i.e., the aggregative form at the early stage is altered during cultivation to the dispersive form at the

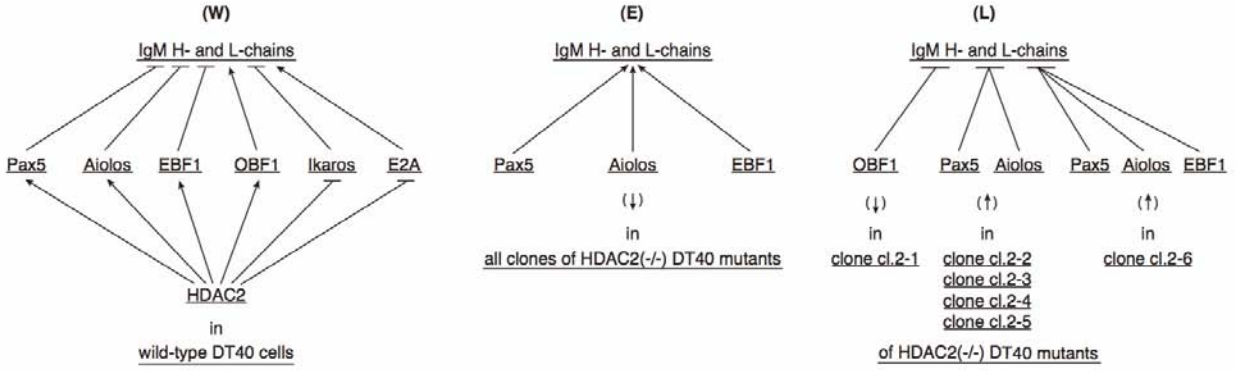

Fig. 6. Ways of controlling gene expressions of IgM H- and L-chains through control of gene expressions of specific transcription factors in DT40 cells, and all or individual clones of HDAC2(-/-) DT40 mutants at early and later stages of continuous cultivation.

In DT40 cells (W), HDAC2 indirectly regulates gene expressions of IgM H- and L-chains through opposite regulation of those of Pax5, Aiolos and EBF1, and Ikaros and E2A. At the early (E) cultivation stage, in all clones of HDAC2(-/-) mutants, IgM H- and L-chains are excessively accumulated depending on their dramatically increased gene expressions caused by drastically decreased gene expressions of Pax5, Aiolos and EBF1, all of which down-regulate gene expressions of the two immunoglobulin proteins. At the later (L) cultivation stage, in individual clones of HDAC2(-/-) mutants, the accumulated IgM H- and L-chains are gradually decreased, which is attributed to their drastically decreased gene expressions in almost the same changing pattern, caused by dramatically increased or decreased gene expressions of Pax5, Aiolos and EBF1 or OBF1 in distinct ways. Clone cl.2-1 seems to be the OBF-1-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 seem to be the Pax5-, Aiolos- and EBF1-dependent type. The figure is a set of figs. 8, 9 and 10 of ref. [58].

later stage, like that of DT40 cells, and it must be normal state for both of DT40 and HDAC2(-/-) mutant cells [42, 58]. 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, 58].

Interestingly, as clearly presented in fig. 4, mRNA levels of various altered transcription factors (and chromatin-modifying enzymes) show 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 and 4) and also in cell morphology [42, 58]. 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, 58], 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 [59], 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 increased until the later stage 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, 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(-)) mutant cells should be more drastic just soon after their generation (birth) by gene targeting techniques. The reasons are as follows. In our previous studies [42, 47, 58] almost all of HDAC2(-/-) (and also Pax5(-)) mutant cells collected at the early stage (3 day-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. Further, we previously reported that the doubling times of the mutant cells are about 12 hours [40, 41, 48].

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. 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 the 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 fig. 6. 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 is not remarkable in all of the six mutant clones (Figs. 3 and 4). Further, detailed participations of altered mRNA levels of PCAF, HDAC7, HDAC9 and Blimp1 in the decreases in protein and mRNA levels of IgM H- and L-chains in HDAC2(-/-) mutant clones during cultivation remain to be resolved, although slight deviations in those of IgM H- and L-chains are detected in both cases of PCAF- and GCN5-deficiencies [41]. Recently, we reported that the IgM H-chain (but not L-chain) gene expression is slightly down-regulated by GCN5-deficiency [60], and the secretory immunoglobulin synthesis is suppressed by PCAF-deficiency [61]. 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. 5). Remarkably, the classification of HDAC2(-/-) mutant clones based on altered transcription factors is coincident with that based on altered members of HATs and HDACs as mentioned above (Figs. 3 and 4). 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 should be obviously distinct from each other in some other cellular characteristics. Naturally, changes in gene expressions of various members of transcription factors, HATs and HDACs, decreasing patterns in protein and mRNA levels of IgM H- and L-chains, and changes in cell morphology during cultivation are obviously different between HDAC2(-/-) and Pax5(-) mutants [47, 58]. These results suggest that the above-mentioned three ways that decrease gene expressions of IgM H- and L-chains in HDAC2(-/-) mutants should be certainly different from those in Pax5(-) mutants.

CONCLUSION

In conclusion, individual clones of HDAC2(-/-) mutants should have the ability to diminish artificially accumulated IgM H- and L-chains (abnormal environment) in distinct ways through various generations (cell divisions) during cultivation. These distinct ways should be definitely caused by diverse changes in gene expressions of transcription factors and chromatin-modifying enzymes (such as Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, Blimp1, OBF1, PCAF, HDAC7, HDAC9 and others) in individual clones of HDAC2(-/-) mutants during cultivation. Of these altered factors and enzymes, Pax5, Aiolos, EBF1 and OBF1 should be 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 propose a brief working hypothesis as follows (Fig. 7). First, the accumulation of IgM H- and L-chains in HDAC2(-/-) mutants should be recognized as an

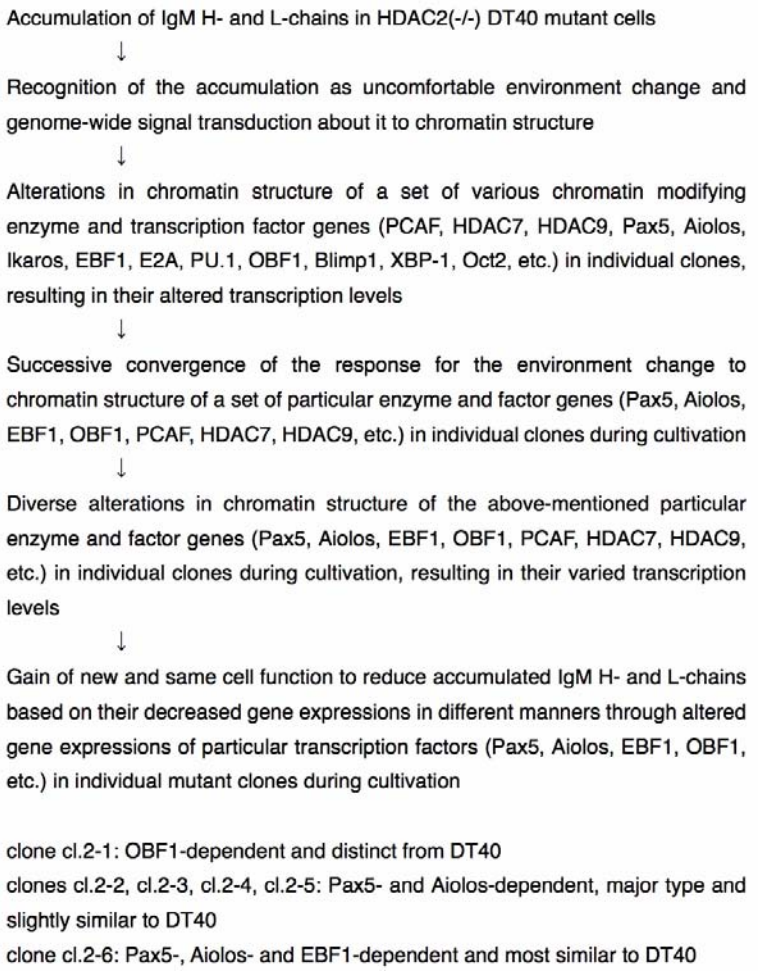

Fig. 7. A proposed model for the ways to exclude IgM H- and L-chains accumulated in HDAC2(-/-) DT40 mutant clones during continuous cultivation. The figure is identical with fig. 11 of ref. [58].

abnormal environment change, and then putative signal(s) concerning it may be genome-widely transmitted to chromatin structure within nucleus, whereas the mechanism and machinery for the processes remain quite unknown. Successively, the environment change should induce alterations in chromatin structure of various transcription factors and chromatin-modifying enzyme genes (such as Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, OBF1, Blimp1, XBP-1, Oct2, PCAF, HDAC7, HDAC9 and other genes), resulting in their altered transcription levels. The response(s) for the environment change should be continuously converged to the chromatin structure of several specific factors and enzyme genes (Pax5, Aiolos, EBF1, OBF1, PCAF, HDAC7, HDAC9 and other genes) in individual mutant clones through various generations during cultivation, and thereby induce diverse alterations in their chromatin structure, resulting in their varied transcription levels. As a result, individual clones of HDAC2(-/-) mutants gain the same and new cell 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.

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