Chapter 3

Generation of Pax5-deficient DT40 mutants, Pax5(-), and protein and mRNA levels of artificially accumulated IgM H- and L-chains in Pax5(-) are rapidly reduced via a lot of generations during continuous cultivation

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SUMMARY

We generated Pax5-deficient DT40 mutant cells, Pax5(-), devoid of the Pax5 gene located on Z chromosome that is monosomy in chickens and analyzed characteristics of Pax5(-) by Western blotting, microscopy, electron microscopy, immuno-electron microscopy and RT-PCR. Protein levels of IgM H- and L-chains in Pax5(-) were drastically increased at the early stage of cultivation and thereafter dramatically decreased during cultivation and at the late stage reached to comparable levels in DT40 cells. Whole and secreted forms of IgM H-chain mRNA in Pax5(-) were dramatically elevated at the first stage and thereafter rapidly reduced until the late stage to comparable levels in DT40 cells. Membrane-bound form of IgM H-chain mRNA and IgM L-chain mRNA were considerably increased at the first stage and thereafter decreased slowly during cultivation. In addition, PCAF and HDAC9 mRNA levels were gradually elevated during cultivation, and HDAC7 mRNA level changed moderately. Aiolos and OBF1 mRNA levels were gradually reduced from the first to late stages, whereas Ikaros and E2A mRNA levels were drastically elevated at the first stage and gradually decreased until the late stage. EBF1 mRNA level was completely decreased at the first stage and remained unchanged during cultivation. PU.1 mRNA level was remarkably reduced at the first stage and gradually elevated until the late stage.

These results, together with our previous findings, revealed not only that Pax5 down-regulates dramatically transcriptions of IgM H- and L-chain genes, which are indirectly regulated by HDAC2 through regulations of gene expressions of Pax5, Aiolos, EBF1, Ikaros and E2A in DT40 cells, but also that during cultivation decreases in elevated protein and mRNA levels of these two immunoglobulin molecules were more rapid in Pax5(-) than in HDAC2(-/-). In addition, influences of Pax5-deficiency on several cellular characteristics should be certainly different from those of HDAC2-deficiency.

INTRODUCTION

Alterations in chromatin structure are strongly involved in regulations of gene expressions, and replication, repair plus recombination of DNA in eukaryotes [1-5], and also concerned in regulations of lymphocyte development and differentiation [6-12]. Among various chromatin modifying enzymes participated in alterations in chromatin structure, histone acetyltransferase(s) (HATs) and deacetylase(s) (HDACs) cooperatively control acetylation and deacetylation levels of particular Lys residues of core histones H2A, H2B, H3 and H4 [13-27]. To assess their individual roles in expressions of cell functions, we have systematically generated a number of homozygous (or conditional) chicken DT40 mutant cell lines, each of which is devoid of a particular member of HDACs and HATs [28-44], by gene targeting techniques [45-55]. Our previous analyses of HDAC2-deficient DT40 mutant cell line, HDAC2(-/-), showed that HDAC2 regulates the amount of IgM H-chain at the steps of transcription of its gene plus alternative processing of its pre-mRNA [28], and down-regulates IgM L-chain gene promoter activity

[31]. Moreover, the HDAC2-deficiency represses transcriptions of HDAC7, Pax5, Aiolos, Ikaros plus EBF1, elevates those of HDAC4, HDAC9, PCAF plus E2A, and changes bulk acetylation levels of several particular Lys residues of core histones H3, H4 and H2B [35]. Therefore, to know individual roles of these altered transcription factors, we subsequently generated and analyzed homozygous DT40 mutant cell lines, EBF1(-/-), Aiolos(-/-), E2A(-/-) and Helios(-/-), devoid of EBF1, Aiolos, E2A and Helios genes, respectively [35, 56-60], and Ikaros-down DT40 mutant cell line, Ikaros(-/-/+), devoid of two alleles of the Ikaros gene located on chromosome 2 that is trisomy in chickens (data will be shown elsewhere). Results obtained showed that EBF1, Aiolos plus Ikaros down-regulate transcriptions of IgM H- and L-chain genes, and E2A up-regulates transcriptions of these two immunoglobulin genes [35]. Taken together, these findings indicated that HDAC2 regulates indirectly gene expressions of IgM H- and L-chains through opposite regulations of transcriptions of EBF1, Aiolos plus Ikaros, and E2A genes [35, 38].

Throughout the process of analyzing several characteristics of HDAC2(-/-) cultivated for different periods, we casually noticed following interesting phenomena represented in our previous article [Chap. 2]. Results obtained by 2D-PAGE, Western blotting and immuno-electron microscopy, together, showed that IgM H- and L-chains are dramatically accumulated at the early stage of cultivation in HDAC2(-/-), and thereafter gradually reduced during cultivation and at the late stage reached to comparable levels in DT40 cells. RT-PCR showed that whole and secreted forms of IgM H-chain mRNA in HDAC2(-/-) 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. Further, RT-PCR carried out on chromatin modifying enzymes and transcription factors revealed that the mRNA level of PCAF in HDAC2(-/-) was drastically elevated from the early to late stages, and those of HDAC7 and HDAC9 changed slightly during cultivation. The mRNA level of EBF1 in HDAC2(-/-) was almost completely reduced at the early stage and thereafter remained unchanged, and that of E2A was slightly elevated at the early stage and remained unchanged. Interestingly, the mRNA level of Pax5 in HDAC2(-/-) was obviously decreased at the early stage and thereafter increased until the late stage. This changing pattern in the Pax5 gene expression was in anti-parallel with those in transcriptions of IgM H- and L-chain genes as a whole. These results revealed not only that the gene expression of Pax5 is certainly controlled by HDAC2, but also that Pax5 is expected to control mainly gene expressions of IgM H- and L-chains among various transcription factors. In addition, immunoblotting using site-specific antibodies for various acetylated Lys residues of core histones (H2A, H2B, H3 and H4) showed that during cultivation bulk acetylation levels of K9, K14, K18, K23 and K27 residues of histone H3 were gradually increased, but those of K5 residue of histone H4 and K16 residue of histone H2B showed different changing patterns.

Next, we studied molecular mechanism of transcription of the Pax5 gene [Chap. 2]. Since detailed

information on the chicken Pax5 gene could not be cited from any database at the start of this study, we directly cloned ~4.9 kb 5'-upstream region of the Pax5 gene from DT40 genomic DNA by our original gene walking techniques. Interestingly, our preliminary chromatin immunoprecipitation (ChIP) assay showed that in HDAC2(-/-) acetylation levels of Lys-9 residues of histone H3 (K9/H3) within some chromatin segments surrounding proximal ~2.0 kb 5'-upstream region of the Pax5 gene were decreased at the early stage but at the late stage restored to comparable levels in DT40 cells. These results qualitatively agreed with the above-mentioned findings on alterations in the Pax5 gene expression, whereas these early and late cultivation stages were convenience sake in fact, since the mutant cells used were already cultivated several times for the use of other experiments.

In this study, we generated and analyzed Pax5-deficient DT40 mutant cell line, Pax5(-), devoid of the Pax5 gene located on Z chromosome that is monosomy in chickens (USCS Genome Browser data base). Protein levels of IgM H- and L-chains in Pax5(-) were drastically increased at the early stage and thereafter rapidly decreased via the middle until late stages to comparable levels in DT40 cells. Whole and secreted forms of IgM H-chain mRNA in Pax5(-) were dramatically elevated at the first stage and thereafter rapidly reduced until the late stage to comparable levels in DT40 cells. Membrane-bound form of IgM H-chain mRNA and IgM L-chain mRNA were considerably increased at the first stage and thereafter decreased until the late stage to comparable levels in DT40 cells. In addition, interestingly, the mRNA levels of PCAF, HDAC7, HDAC9, Aiolos, OBF1, Ikaros, E2A, EBF1 and PU.1 in Pax5(-) changed in different patterns during cultivation. These results, together with others, indicated not only that Pax5 should be certainly different from HDAC2 in the participation in transcriptions of IgM H- and L-chains, but also that Pax5(-) must be obviously distinct from HDAC2(-/-) in several cellular properties.

RESULTS

Generation of Pax5-deficient DT40 mutant cells, Pax5(-)

To generate the Pax5-deficient DT40 mutant cell line, we transfected the targeting ΔPax5/hyg vector into DT40 cells. After integration of the targeting vector into one Pax5 allele, three stable transfectant clones (cl.46, cl.58 and cl.62) were selected in the presence of hygromycin (hyg) based on the positive 11.5 kb SacI fragment hybridized with probe Pax5 (Fig. 3-1A). Surprisingly, all of these transfectants lost completely the endogenous 7.8 kb SacI fragment at the same time (Fig. 3-1B). To verify whether or not the Pax5 gene was really disrupted in these mutant clones, we carried out RT-PCR using appropriate primers specific for Pax5 on total RNAs. The mRNA level of Pax5 was not detected in three independent mutant clones (cl.46, cl.58 and cl.62), whereas in three Pax5(+) clones (cl.1, cl.2 and cl.3) having the endogenous Pax5 gene and randomly integrated hyg-gene, it was comparable to that in DT40 cells (Fig. 3-2). These results indicated that the Pax5 gene exists as a single allele and agreed with the

fact that the Pax5 gene is located on Z chromosome, which is monosomy in chickens as mentioned above, distinct from its some homologues located on other chromosomes. Hereafter, we regarded these three transfectants as the Pax5-deficient DT40 mutant cells, Pax5(-).

Pax5-deficiency affects severely or moderately on gene expressions of IgM H- and L-chains, chromatin modifying enzymes and transcription factors

We studied effects of the Pax5-deficiency on several cellular characteristics as follows. Three independent Pax5(-) clones (cl.46, cl.58 and cl.62) proliferated and reached to a maximum cell density comparable to that of DT40 cells, suggesting that the Pax5-deficiency had a slight effect on growth rate and maximum cell density (Fig. 3-1C). To know whether or not the Pax5-deficiency affects on gene expressions of IgM H- and L-chains, chromatin modifying enzymes and transcription factors, we carried out RT-PCR, using primers IgM Hc, IgM Hs plus IgM Hm and IgM L (which are specific for whole, secreted plus membrane-bound forms of IgM H-chain mRNA and IgM L-chain mRNA), and also primers specific for PCAF, HDAC2, HDAC4, HDAC7, HDAC9, Ikaros, Aiolos, E2A, EBF1 and CstF-64, on total RNAs prepared from three Pax5(-) clones (cl.46, cl.58 and cl.62), together with DT40 (W) and three Pax5(+) clones (cl.1, cl.2 and cl.3) as control (Fig. 3-2). In all three Pax5(-) clones, the levels of whole and secreted forms of IgM H-chain mRNA were dramatically elevated, whereas those of membrane-bound form of IgM H-chain mRNA and IgM L-chain mRNA were slightly and considerably elevated. Concerning chromatin modifying enzymes, in three Pax5(-) clones, the mRNA levels of PCAF and HDAC9 were dramatically increased, that of HDAC7 was slightly reduced, and those of HDAC2 and HDAC4 remained unchanged. On the other hand, regarding transcription factors, the mRNA levels of Ikaros and E2A were slightly elevated but that of Aiolos mRNA was slightly decreased. Interestingly, the mRNA level of EBF1 was almost completely reduced in all of three Pax5(-) clones, whereas that of CstF-64 unchanged. These results indicated that Pax5 exhibits severe effects on gene expressions of IgM H- and L-chains, and also those of EBF1, PCAF and HDAC9, but moderate effects on those of residual enzymes and factors examined.

Artificially accumulated IgM H- and L-chains in Pax5(-) mutant cells are gradually reduced during continuous cultivation

We studied changing patterns in several properties of Pax5(-) clones (cl.46, cl.58 and cl.62) at the first (F; 4 days), early (E; 8 days), middle (M; 13 days) and late (L; 20 days) stages of cultivation. These cultivation stages were practically counted from the first stock day and at shorter intervals than corresponding those employed for HDAC2(-/-) mutants [Chap. 2]. To know changing patterns in cellular protein levels, we first analyzed total cellular proteins prepared from Pax5(-) clone (cl.58) at the early and late stages by 2D-PAGE. The amounts of IgM H- and L-chains were dramatically and

certainly elevated at the early stage, and thereafter at the late stage reduced to almost similar levels in DT40 cells (data not shown). Western blotting, using antibody for chicken IgM L-chain that cross-reacts with IgM H-chain, revealed that protein levels of IgM H- and L-chains (detected as two bands) were dramatically increased at the early stage in three Pax5(-) clones (cl.46, cl.58 and cl.62), and thereafter gradually reduced via the middle to late stages to comparable levels in DT40 cells (W) (Fig. 3-3). Electron microscopy showed that electron-dense materials were accumulated in irregularly elaborated and vesiculated rough endoplasmic reticula in Pax5(-) at the early stage, but not at the late stage like in DT40 cells (Fig. 3-4A). Immuno-electron microscopy revealed not only that colloidal gold immuno-labeling for chicken IgM H-chain was vividly observed in electron-dense materials accumulated in the dilated and vesiculated rough endoplasmic reticula only at the early stage, but also that the heightened protein levels were obviously reduced at the late stage to almost similar levels in DT40 cells (Fig. 3-4B). These results, together, indicated that protein levels of IgM H- and L-chains in Pax5(-) are dramatically and obviously accumulated at the early stage of cultivation, and thereafter gradually reduced during cultivation and at the late stage reached to comparable levels in DT40 cells.

Morphological property of Pax5(-) mutant cells slightly changes during continuous cultivation

Microscopy showed that Pax5(-) clone (cl.62) at both the early and late cultivation stages was morphologically observed to be dispersive form like that of DT40 cells, in any distinct ranges of vision within microscopy and their magnified visions (Suppl. Fig. 3-S1). In addition, Pax5(-) was observed to be mature plasma B cell-like form at the early stage but thereafter to change to normal form at the late stage, like that of DT40 cells. The morphology of Pax5(-) and its changing patterns during cultivation were clearly different from those of HDAC2(-/-) (data will be shown in [Chap. 4]).

Gene expressions of IgM H- and L-chains in Pax5(-) mutant cells change during continuous cultivation

To clarify changing patterns in gene expressions of IgM H- and L-chains in Pax5(-) during cultivation, we carried out RT-PCR, using appropriate primers IgM Hc, IgM Hs, IgM Hm and IgM L, on total RNAs prepared from three Pax5(-) clones (cl.46, cl.58 and cl.62) at the first, early, middle and late cultivation stages (Fig. 3-5). Whole and secreted forms of IgM H-chain mRNA in Pax5(-) were dramatically increased at the first stage, and thereafter rapidly reduced via the early and middle stages and at the late stage reached to almost the same levels in DT40 cells. On the other hand, membrane-bound form of IgM H-chain mRNA and IgM L-chain mRNA were considerably increased at the first stage, and thereafter gradually and slowly decreased via the early and middle stages and at the late stage reached to almost the same levels in DT40 cells.

Gene expressions of HDAC9 plus PCAF or HDAC7 in Pax5(-) mutant cells dramatically or moderately change during continuous cultivation

We carried out RT-PCR, using various appropriate primers specific for chromatin modifying enzymes; HDAC1, HDAC2, HDAC3, HDAC4, HDAC7, HDAC8, HDAC9, PCAF, GCN5, HAT1, Elp3, p300, MORF, MOZ and TIP60, on total RNAs prepared from three Pax5(-) clones at the first, early, middle and late cultivation stages (Fig. 3-6A). Gene expressions of HDAC9 and PCAF in Pax5(-) were dramatically increased from the first via early and middle to late stages. The gene expression of HDAC7 was slightly decreased at the first stage but thereafter slightly increased until the late stage to almost similar level in DT40 cells. On the other hand, gene expressions of residual HATs and HDACs tested remained unchanged during cultivation.

Gene expressions of Aiolos, Ikaros, EBF1, E2A, PU.1, Blimp1 and OBF1 in Pax5(-) mutant cells differently change during continuous cultivation

We carried out RT-PCR, using various appropriate primers specific for transcription factors, such as Oct1, Oct2, OBF1, NF-kB, RelB, YY1, NF-AT, Blimp1, XBP-1, EBF1, PU.1, E2A, Aiolos, Ikaros and CstF-64, on total RNAs prepared from three Pax5(-) clones at the first, early, middle and late cultivation stages (Fig. 3-6B). Gene expressions of Aiolos and OBF1 in Pax5(-) were gradually reduced from the first via early and middle to late stages to undetectable levels. On the other hand, gene expressions of Ikaros, E2A and Blimp1 were dramatically increased at the first stage and thereafter gradually decreased via the early and middle stages and finally at the late stage reached to almost same levels in DT40 cells. The gene expression of EBF1 was completely decreased at the first stage and remained unchanged as undetectable level until the late stage. The gene expression of PU.1 was remarkably reduced at the first stage and gradually increased at the late stage to slightly higher level than that in DT40 cells. The relative mRNA levels of these altered transcription factors, together with those of altered chromatin modifying enzymes, are shown in Supplementary Figure 3-S2.

DISCUSSION

In this study, to explore the participation of Pax5 in gene expressions of IgM H- and L-chains, we first generated and analyzed the Pax5-deficient DT40 mutant cell line, Pax5(-), devoid of the Pax5 gene (as a single allele) located on Z chromosome that is monosomy in chickens (Fig. 3-1). Results obtained from Pax5(-) (Fig. 3-2), together with those from chicken DT40 mutant cell lines, EBF1(-/-), Aiolos(-/-), Helios(-/-), E2A(-/-) and Ikaros(-/-/+), which were generated by us, revealed that Pax5, Ikaros, EBF1 and Aiolos down-regulate transcriptions of IgM H- and L-chain genes, and E2A up-regulates transcriptions of these two immunoglobulin genes [35, 38, 58]. Thus, these results, combined with those from

HDAC2(-/-), indicated that HDAC2 regulates indirectly gene expressions of IgM H- and L-chains through controlling those of Pax5, Ikaros, EBF1 plus Aiolos and that of E2A in opposite manners [35, 38, Chap. 2]. Surprisingly, as shown in [Chap. 2], our qualitative 2D-PAGE, Western blotting, immuno-electron microscopy and/or RT-PCR analyses of HDAC2(-/-) clones (cl.33-12, cl.33-28, cl.33-30 and cl.45-28) cultivated for different cultivation periods revealed not only that protein and mRNA levels of IgM H- and L-chains are dramatically and considerably accumulated at the early (~10 days) stage of cultivation, but also that these accumulated immunoglobulin proteins and mRNAs are obviously reduced at the late (~60 days) cultivation stage to comparable levels in DT40 cells. In addition, gene expressions of HDAC7, HDAC9, PCAF, EBF1, E2A, Pax5 and Aiolos each were certainly altered in different patterns in HDAC2(-/-) mutants during cultivation. Interestingly, the changing pattern in the gene expression of Pax5 in HDAC2(-/-) during cultivation was anti-parallel with those in gene expressions of IgM H- and L-chains, whereas those in gene expressions of residual altered factors (such as EBF1, E2A plus Aiolos and others) were neither parallel nor anti-parallel so much. These results, together with others [35, 38], suggested that Pax5 should control preferentially gene expressions of IgM H- and L-chains among various transcription factors tested.

Therefore, we next studied several characteristics of Pax5(-) clones (cl.46, cl.58 and cl.62), which were cultivated for several distinct periods, by Western blotting, RT-PCR, microscopy, electron microscopy and immuno-electron microscopy. Protein levels of IgM H- and L-chains in Pax5(-) were drastically and considerably increased at the early (8 days) stage of cultivation, and thereafter gradually decreased via the middle (13 days) until late (20 days) stages to comparable levels in DT40 cells (Figs. 3-3 and 3-4). Concerning mRNA levels of these two immunoglobulin proteins, whole and secreted forms of IgM H-chain mRNA were dramatically elevated at the first (4 days) stage (prior to the early stage), and thereafter these accumulated two type mRNAs were rapidly reduced until the late stage to comparable levels in DT40 cells (Fig. 3-5). Membrane-bound form of IgM H-chain mRNA and IgM L-chain mRNA were considerably increased at the first stage, and thereafter slowly decreased until the late stage to almost the same levels in DT40 cells. Thus, changing patterns in protein and mRNA levels of IgM H- and L-chains in Pax5(-) during cultivation were similar to those in HDAC2(-/-) on the whole, however, these changing speeds in Pax5(-) were obviously observed to be faster compared with those in HDAC2(-/-) [see Chap. 2]. In addition, Pax5(-) was observed to be dispersive form at both the early and late stages, like that of DT40 cells (Suppl. Fig. 3-S1), whereas HDAC2(-/-) was seemed to be aggregative form at the early stage but dispersive form at the late stage (data will be shown in [Chap. 4]).

The mRNA levels of PCAF and HDAC9 in Pax5(-) were gradually elevated from the first to late stages, and that of HDAC7 changed moderately during cultivation (Fig. 3-6A and Suppl. Fig. 3-S2). These changing patterns were similar to those observed in HDAC2(-/-) as a whole [see Chap. 2]. On the other hand, concerning several transcription factors, Pax5(-) showed different changing patterns from

those in HDAC2(-/-) as follows (Fig. 3-6B and Suppl. Fig. 3-S2). The mRNA levels of Aiolos and OBF1 were gradually reduced from the first to late stages to undetectable levels, and those of Ikaros, E2A and Blimp1 were drastically elevated at the first stage and thereafter gradually decreased until the late stage to almost same or slightly higher levels in DT40 cells. The EBF1 mRNA level was completely decreased at the first stage and remained unchanged as undetectable level throughout cultivation. The PU.1 mRNA level was remarkably reduced at the first stage and gradually elevated until the late stage: the level being slightly higher than that in DT40 cells.

In summary, the reason for the differences in the changing speeds of protein plus mRNA levels of IgM H- and L-chains between Pax5(-) and HDAC2(-/-) is probably based on the fact that Pax5 should participate at down-stream of HDAC2 in regulation of gene expressions of these two immunoglobulin proteins [35, 38]. In addition, both of the above-mentioned differences and those in the cell morphology between Pax5(-) and HDAC2(-/-) during cultivation must be due to diverse alterations in gene expressions of particular transcription factors; Pax5, Aiolos, OBF1, Ikaros, E2A, EBF1, Blimp1, PU.1 and so on, but not to alterations in gene expressions of chromatin modifying factors; PCAF, HDAC7 and HDAC9 and so on. As a result, Pax5(-) mutants should be obviously different from HDAC2(-/-) mutants in several cellular characteristics.

METHODS

Cell cultures

DT40 and Pax5(-/-) mutant cells were grown as described [28, 35, Chap. 2]. Cell numbers were counted at indicated times to determine growth rate. Pax5(-) mutant clones were continuously cultivated and collected at indicated periods, i.e., at the first (F; 4 days), early (E; 8 days), middle (M; 13 days) and late (L; 20 days) stages of cultivation. These cultivation stages were practically counted from the first stock day, although all positive mutant clones were picked up on 96-well plates at ~11 days after knockout of one Pax5 allele, cultivated for another several days to be ~10^6 cells/ml and stocked at -80°C until use. DT40 (W) was used as control.

Generation of Pax5-deficient DT40 cells, Pax5(-)

In this study we used only the cassette carrying hyg transcribed by the chicken β-actin promoter [61] in fact, since the Pax5 gene is located on Z chromosome that is monosomy in chickens (USCS Genome Browser data base), though two other cassettes carrying hisD or neo had also been prepared. Partial genomic Pax5 fragment was obtained from DT40 genomic DNA by PCR using appropriate primers synthesized based on nucleotide sequences from a database, and those of the PCR amplified product were confirmed by the PCR sequencing protocol as described [32, 35].

We generated the ΔPax5/hyg vector for disruption of the Pax5 gene as follows. The 5'-arm, a HindIII digested 1.8 kb PCR fragment (obtained using sense primer 5'-GGTGTGAACCAGCTGGGGGGCGTTTTTGTG-3' from exon 2 and antisense primer 5'-TGGGCACGGTGTCGTTATCGCACACTCGTT-3' from exon 3), and the 3'-arm, a 4.7 kb PCR fragment (obtained using sense primer 5'-ACCGGGAGCATTAAGCCTGGAGTGATTGGA-3' from exon 3 and antisense primer 5'-AGACTGGGACTTGCTGATTGGGCGGCTGCT-3' from exon 4) were ligated to the pBluescript II vector. The hyg cassette flanked by a loxP site [29] was inserted between the 5'-arm and 3'-arm (5'-upstream and 3'-downstream fragments). In the resultant targeting vector, therefore, the genomic DNA sequences corresponding to exon 3 of the Pax5 gene were replaced with the hyg-carrying cassette.

To obtain Pax5-deficient mutant cells, we transfected the ΔPax5/hyg vector into DT40 cells and selected three hyg-resistant transfectants in medium containing 2.5 mg of hyg per ml [29]. Southern blotting showed that all of these three transfectants carrying the hyg cassette had positive 11.5 kb fragment hybridized with probe Pax5 and lost completely the endogenous positive 7.8 kb fragment at the same time. Therefore, we regarded these mutants as the Pax5-deficient DT40 mutant cells, Pax5(-).

RT-PCR

Total RNAs were isolated from exponentially growing DT40 cells (W) and mutant cells as described [28, 35]. Mutant cells were three wild-type Pax5(+) clones (cl.1, cl.2 and cl.3) containing the Pax5 allele plus hyg cassette, and three Pax5 (-) mutant clones (cl.46, cl.58 and cl.62) at the first (F; 4 days), early (E; 8 days), middle (M; 13 days) and late (L; 20 days) stages of cultivation. Total RNAs obtained were subjected to synthesize first-strand cDNA using a kit (ReverTra Ace-α-™). The mRNA levels were measured by RT-PCR, using equal amounts of total RNAs and appropriate primers listed in our previous reports [32, 35, 62]. 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 [32, 35]. Data analyses were carried out by Multi Gauge Ver3.X software using a luminescent image analyzer LAS-1000plus (FUJIFILM). Data calibrated with internal control in three Pax5(-) clones are indicated as percentages of control values obtained from DT40 cells. Chicken glyceraldehydephosphate dehydrogenase (GAPDH) was used as control.

Southern blotting

Genomic DNAs were isolated from three Pax5(-) mutant clones, one Pax5(+) clone and DT40 cells, digested with the indicated enzyme (SacI), separated in 0.8% agarose gel, electro-transferred to Hybond N+ membrane, and hybridized with ^{32}P-labeled probe Pax5 as described [28]. The probe Pax5, corresponding to the 5'-end region of intron 4 (and 3'-outer side of exon 4) of the Pax5 gene, comprised

the 0.47 kb fragment obtained by PCR using sense primer 5'-CCAGTCACAGCATAGGTGA-3' and antisense primer 5'-TGGCTCGAGATCGCAAAGT-3' from intron 4.

Western blotting

Western blotting was performed as described [28, 35, Chap. 2]. Whole cellular proteins were prepared from DT40 and Pax5(-) mutant clones (cl.46, cl.58 and cl.62) at the early, middle and late stages of cultivation, with intervals shorter than corresponding those for HDAC2(-/-) [see Chap. 2]. In brief, cells (1 x 10^7) were treated with 10% trichloroacetic acid and lysed in 100 μl of SDS buffer. Aliquots (10 μl) of 1:50 dilutions of the resultant cell extracts were subjected to 12% SDS-PAGE and electro-transferred to membranes, and proteins were detected with rabbit anti-chicken IgM L-chain antiserum (as primary antibody) that cross-reacts with IgM H-chain. β-actin was used as control. Antibodies used were: A30-100A (Bethyl Laboratories Inc., TX, USA) for IgM H- and L-chains and ab6276 (Abcam) for β-actin. The relative amounts of IgM H-chain and large (high) and small (low) forms of IgM L-chain were measured as described [32, 35].

Microscopy, electron microscopy and immuno-electron microscopy

Pax5(-) mutant clones (cl.46, cl.58 and cl.62) at the early and late stages of cultivation and DT40 cells were exponentially growing. Microscopy was carried out in distinct ranges of vision with magnified visions as described [36, 56, 57]. Electron microscopy and immuno-electron microscopy using immuno-gold labeling were carried out as described [63, Chap. 2]. Exponentially growing DT40 and Pax5(-) clone cl.58 at the early and late stages 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) for 60 min. After rinsing in PBS, the sections were incubated with biotinylated horse anti-goat IgG (Vector Laboratories, Burlingame, CA, USA; diluted 1:200 with 1% BSA in PBS) for 40 min. After washing with PBS, the sections were incubated with 8 nm colloidal gold conjugated anti-biotin antibody (Vector Laboratories, Burlingame, CA, USA; diluted with 1% BSA in PBS) for 30 min. After washing with distilled water and drying, the sections were contrasted with 2% uranyl acetate in 70% methanol and Reynolds' lead citrate and observed in a JEOL 1200EX transmission electron microscope operating at 80

kV (JEOL, Tokyo, JAPAN). As control, primary antibody was omitted or replaced by normal goat serum.

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FIGURE LEGENDS

Figure 3-1. Genomic organization of chicken Pax5 gene and generation of Pax5-deficient DT40 mutant cells, Pax5(-)

A. Schematic representation of the chicken Pax5 gene locus (top) with enlarged drawing of the targeted region (middle) and its targeted allele (bottom). Locations of exons are indicated by solid boxes with appropriate designations. White box indicates drug resistance cassette (hyg). Location of probe Pax5 is indicated by a gray box. Only relevant restriction sites are indicated. Possible relevant fragments obtained from SacI digestion are shown with their length in kb.

B. Southern blotting of homologous recombination event. Genomic DNAs were prepared from DT40, one mutant clone cl.1(+) integrated randomly with ΔPax5/hyg construct and three homozygous Pax5(-) mutant clones cl.46(-), cl.58(-) and cl.62(-). The SacI fragments were analyzed with probe Pax5.

C. Growth rate of Pax5(-) mutant cells. Pax5(-) mutant clones cl.46(-), cl.58(-) and cl.62(-) and DT40 cells 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 mutant clones are shown in the figure.

Figure 3-2. Effects of Pax5-deficiency on gene expressions of IgM H- and L-chains, and several members of HATs, HDACs and transcription factors

Total RNAs were extracted from DT40 cells (W), three Pax5(+) clones (cl.1, cl.2 and cl.3) randomly integrated with ΔPax5/hyg construct and three homozygous Pax5(-) clones (cl.46, cl.58 and cl.62). RT-PCR was performed using equal amounts of total RNAs and appropriate primers for whole IgM H-chain mRNA (IgM Hc), its secreted form (IgM Hs), its membrane-bound form (IgM Hm), IgM L-chain mRNA (IgM L) and also mRNAs of several members of HATs, HDACs and transcription factors (indicated by appropriate designations). Chicken GAPDH was used as control.

Figure 3-3. Alterations in amounts of IgM H- and L-chains in Pax5(-) DT40 mutant cells during cultivation

Total cellular proteins were prepared from Pax5(-) mutant clones cl.46(-), cl.58(-) and cl.62(-) at the early (E; 8 days), middle (M; 13 days) and late (L; 20 days) cultivation 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 filter, and proteins were detected with anti-chicken IgM L-chain antiserum (as primary antibody) that cross-reacts with IgM H-chain. Chicken β-actin was used as internal control. Typical patterns are shown in the upper panel. Relative levels of IgM H-chain and large (high) plus small (low) forms of IgM L-chain are shown in the lower panel. Symbols for IgM H- and L-chains are shown in the bottom.

Figure 3-4. Alterations in amounts of IgM H-chains in Pax5(-) DT40 mutant cells during cultivation

Pax5(-) mutant cells (cl.58) collected at the early (E; 8 days) and late (L; 20 days) cultivation stages and DT40 cells (W) were fixed. Electron microscopy (A) and immuno-electron microscopy using anti-chicken IgM H-chain antiserum (B) were carried out. Dense cytoplasmic fraction (indicated by arrow) due to accumulated IgM H-chain was observed only at the early (E) cultivation stage of Pax5(-) (E in A). A large number of IgM H-chain proteins were observed only at the early (E) cultivation stage of Pax5(-) (E in B).

Figure 3-5. Alterations in gene expressions of IgM H- and L-chains in Pax5(-) DT40 mutant cells during cultivation

Total RNAs were extracted from three Pax5(-) mutant clones cl.46(-), cl.58(-) and cl.62(-) at the first (F; 4 days), early (E; 8 days), middle (M; 13 days) and late (L; 20 days) cultivation stages and DT40 cells (W). RT-PCR was performed using equal amounts of total RNAs and appropriate primers for Pax5, whole IgM H-chain mRNA (IgM Hc), its secreted form (IgM Hs), its membrane-bound form (IgM Hm) and IgM L-chain (IgM L). Chicken GAPDH was used as control. Typical patterns are shown in the upper panel. Relative mRNA levels of whole, secreted and membrane-bound forms of IgM H-chain and IgM L-chain are shown in the lower panel. Symbols for mutant clones are shown in the bottom.

Figure 3-6. Alterations in gene expressions of HDACs, HATs and transcription factors in Pax5(-) DT40 mutant cells during cultivation

Total RNAs used were the same ones as in Fig. 3-5. 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. 3-5.

Supplementary Figure 3-S1. Morphology of Pax5(-) DT40 mutant cells during cultivation

Microscopy on Pax5(-) mutant cells at the early (E) and late (L) cultivation stages and DT40 cells (W) was performed in distinct ranges of vision with magnified visions.

Supplementary Figure 3-S2. Relative mRNA levels of altered genes in Pax5(-) DT40 mutant cells during cultivation

Relative mRNA levels of altered genes in Fig. 3-6 are shown. Symbols for Pax5(-) mutant clones are shown in the right.