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REVERSIBLE LYMPHOMAGENESIS IN CONDITIONALLY C-MYC EXPRESSING MICE

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It is well documented that deregulation of MYC leads to tumor development, yet many aspects of this process are only partially understood. We have established a transgenic mouse model in which c-MYC is conditionally expressed in lymphoid cells using the tetracycline-regulated system of gene regulation. Mice with continuously expressed transgenic c-MYC died of invasive T- or B-cell lymphomas within 4 months. Lymphomas developing in transgenic mice were c-MYC dependent since doxycycline treatment led to tumor regression. Using transplantation of established tumor cell lines labeled with GFP, we followed the fate of neoplastic cells in recipients upon MYC inactivation. This approach allowed us to elucidate both apoptosis and differentiation as mechanisms of tumor elimination. Comparative genomic hybridization (CGH) and FISH analyses were performed in order to analyze possible chromosomal aberrations induced by c-MYC. We observed that overexpression of c-MYC is sufficient to induce recurrent patterns of genomic instability. The main observation was a gain of genomic material that corresponded to chromosome 15 in several T-cell tumors, which could be identified as trisomy.

Key words: *MYC; lymphomas; genomic instability*

Since the first description as the cellular homologue of the transforming sequences of the avian myelocytomatosis retrovirus,[1] many properties of the c-MYC (MYC*)* protooncogene have been described. The product of MYC oncogene is a bHLHZip transcription factor that is conserved from Xenopus to human.[2] Owing to the presence of the HLH and Zip motifs, MYC is able to participate in various protein-protein interactions.[3–7] The prototype MYC transcription factor forms a heterodimer with the related protein MAX.[8,9] It has been shown that both activation and repression of transcription could be mediated by MYC.[10] In general, MYC expression correlates tightly with the proliferative potential of a cell: in quiescent cells, expression is virtually undetectable, whereas upon mitogen stimulation, there is a rapid and transient burst in mRNA and protein levels.[11] It seems that MYC plays a role in cell cycle regulation, differentiation, apoptosis, metabolism, cell adhesion and regulation of hematopoietic homeostasis.[12–16]

Expression of MYC is altered in a wide variety of human and animal tumors including breast, colon and cervical carcinomas, small cell lung carcinomas, osteosarcomas, glioblastomas, myeloid leukemias and lymphomas.[17] MYC is activated in these tumors by a variety of genetic alterations, *e.g.*, chromosomal translocations, retroviral transduction, proviral insertion and gene amplification. Several transgenic models confirmed that MYC overexpression leads to tumorigenesis in different tissues.[18–22] Results obtained with these transgenic models suggested that the effect of MYC on cell fate depends on cell type, stage of differentiation and physiological environment.

Genomic instability is frequently observed in a diversity of tumors and correlates with the selection of the malignant phenotype.[23] Genome wide analysis techniques, such as chromosome painting, comparative genomic hybridization, representational difference analysis and restriction landmark genome scanning, have revealed that certain oncogenes might induce genome instability.[24,25] *In vitro* studies made on Rat1A, Ba/F3 and primary human fibroblast cells showed that MYC might also contribute to tumorigenesis by destabilizing the cellular genome.[26–28]

In order to study the effects of MYC activation in lymphoid tissues, we generated a conditional mouse model based on the tet-off system. These mice develop both T- and B-cell lymphomas. Upon transgene inactivation, tumor cells not only enter apoptosis but also show signs of differentiation. Furthermore, we demonstrate that overexpression of MYC alone can induce genomic instability.

MATERIAL AND METHODS

Generation of transgenic mice

An EcoRI-HindIII fragment that contained the human c-MYC cDNA (exons 2 and 3, provided by M. Eilers) was inserted into the bidirectional tetracycline-dependent expression vector pBI5,[29] creating tetO-MYC; μE-tTA mice used in our study were previously described.[30] In both cases, founders were derived in the NMRI outbread background. The genotype of the transgenic animals was determined by Southern blot and/or PCR. Animal studies were approved by governmental review boards.

Luciferase activity measurement

Tissues from tTA/MYC mice were homogenized in extraction buffer (100 mM K-PO_4, 1% Triton X, 10 % glycerin, 2 mM EDTA and protease inhibitors) and luciferase enzyme activities were measured using luciferase buffer from Promega (Madison, WI) in a luminometer (Lumat LB 9507, Berthold Technologies, Germany).

Histology

Tissues were embedded in Tissue-Tek O.C.T. Compound (Sakura, The Netherlands), snap frozen in liquid nitrogen and stored at −80°C. Cryostat sections of 4 μm were prepared, fixed with acetone and stained with hematoxylin and eosin to investigate tumor invasion. Apoptotic cells were detected using the Tunel Assay Kit (Promega) according manufacturer's instructions. Conventional Giemsa staining was used to analyze bone marrow, blood and tumor cell lines.

For the analysis of GFP expression, tissues were first fixed for 24 hr in 4% buffered formalin with 7% picric acid and 10%

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sucrose and then embedded in Tissue-Tek O.C.T. Compound. Nuclei were stained with fluorescent dye (DAPI, 0.1 μg/ml; Roche, Switzerland).

All slides were analyzed using a Leica microscope (DMIRB/E) and OpenLab software (version 3.1.1.). The excitation wavelength was 470 nm for GFP and 359 nm for DAPI.

FACS analysis

Single cell suspensions from the tissues were depleted of red cells using Lympholyte-M (Cederlane, Canada). For FACS analysis cells were blocked with anti-FcγII/III receptor antibody and stained with different combinations of CD3-FITC, TCRβ-biotin, B220-PE, CD8-FITC, CD4-PE, IgD-FITC, IgM-PE, CD43-biotin, CD23-PE, CD21-biotin, CD5-APC and Mac-1-PE antibodies (all produced by Pharmingen, Heidelberg, Germany). Biotin-labeled antibodies were visualized using streptavidin-CyChrome (Pharmingen). The annexin V-FITC apoptosis detection kit was obtained from Pharmingen and Annexin V and PI staining were performed according to the manufacturer instruction. Cell surface marker expression was analyzed using a 4-color flow cytometer (FACS-Calibur) and Cell Quest software (Becton Dickinson, Heidelberg, Germany).

Tumor cell lines

Tumors were prepared as single cell suspensions and adapted to *in vitro* growth in RPMI1640 supplemented with 10% FCS, penicillin/streptomycin, NEAA, glutamine and beta-mercaptoethanol (all from PAN Biotech, Germany). Tumor cell line proliferation was measured by counting Trypan blue negative cells.

Doxycycline treatment

To suppress expression of MYC transgene, mice were given doxycycline (ICN Biomedicals, Inc., Ohio) in the drinking water at a concentration of 100 μg/ml. Tumor cell lines were treated with 2 μg/ml of doxycycline for the indicated time periods.

Tumor transfers

Tumor tissues were prepared as a single cell suspension in PBS and 10^7 cells were inoculated *i.v.* or *i.p.* into syngeneic mice. Alternatively, some tumors were first adapted to *in vitro* growth and infected with a retrovirus expressing GFP prior to injection.

CGH (Comparative Genomic Hybridization)

DNAs from tumor cell lines and normal mouse tissue, as reference DNA, were used. CGH was performed as previously described.[31,32] The tumor cell line DNA was labeled with biotin-16-dUTP and normal reference DNA with digoxygenin-11-dUTP. Labeled DNAs, each 1 μg, were precipitated with 70 μg unlabeled Cot1-DNA and 30 μg herring sperm DNA. The precipitate was dissolved in hybridization buffer, denatured at 76°C for 6 min and hybridized after pre-annealing for 3 days on normal denatured metaphase spreads. After incubation the detection was performed with avidin-Texas-Red and anti-digoxygenin-FITC. Counterstaining was done with DAPI.

The ratio of green to red fluorescence intensities along each chromosome was calculated by the V3.05 version of Isis software (Metasystems, Heidelberg, Germany). Between 10 and 15 metaphases were analyzed per sample to ascertain copy number changes for each cell line. A copy number change was considered a gain when the cell line to normal (red to green fluorescence) ratio exceeded the 1.25 cut off level and as a loss when the ratio was below 0.8.

FISH (Fluorescence in situ *hybridization)*

Chromosomes of the cell lines were prepared by standard techniques. Whole chromosome paints (WCP) were made according to the manufacturer's protocol (Cambio, Ltd., Cambridge, UK). All BAC probes were from RPCI23 or RPCI24 libraries and purchased at the BACPAC resource center (www.chori.org/bacpac). For fluorescence in situ hybridization (FISH), standard techniques were applied.

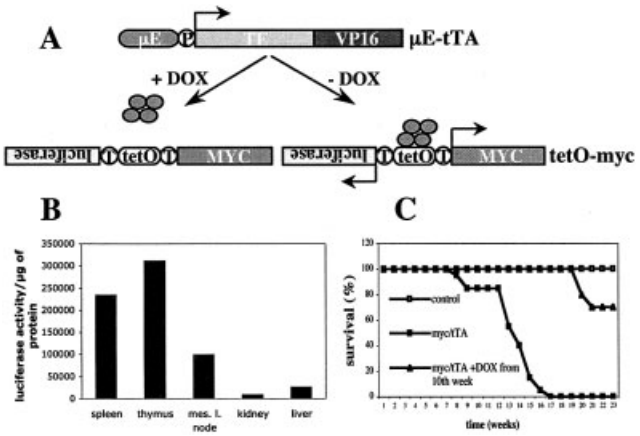

FIGURE 1 – Induction of lymphomas by MYC under the control of tet-off system. (*a*) Constructs for MYC expression in transgenic mice. The tetracycline-dependent transactivator (the tet-off system) and the tet-operator Myc-cassette (with bidirectional promoter expressing also luciferase) are indicated. (*b*) Adult tTA/MYC mice were sacrificed and luciferase activities in indicated tissues were measured. Representative results are shown. (*c*) Survival rate of transgenic animals. Life span of at least 20 animals per each group was monitored. Single transgenic or wild-type animals were used as a control. One group of tTA/MYC mice was given doxycycline in the drinking water (100 μg/ml) starting from 10th week of age (time when typically first signs of disease are observed).

Analysis of mRNA expression levels

Total RNA was isolated from tumor cell lines, thymocytes and $B220^+$splenocytes using High Pure RNA Isolation Kit (Roche, Switzerland) and reverse transcribed with M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA) and random hexamer primers (Roche). In order to distinguish between human and mouse MYC transcript, primers specific for both species were created and used in PCR. For other genes from chromosome 15 specific PCR primers were designed (all primers sequences are available upon request). β-actin primers were used as a control for a housekeeping gene.

RESULTS

MYC overexpression in the lymphoid lineage causes T- and B-cell lymphomas

We generated a mouse model for studying the effects of conditional MYC expression in lymphoid cells using the tet-off system.[29,33] Two types of transgenic mice were established (Fig.1*a*). The first line (μE-tTA) expresses the tetracycline-transactivator (tTA) under the control of the intronic Ig-μ heavy chain enhancer (μE) and a minimal promoter.[30,34] The second line (tetO-MYC) contains the human MYC cDNA and a luciferase reporter gene under the control of the tetracycline responsive bidirectional promoter (tetO).[29] In double transgenic animals (tTA/MYC), tTA mediates the transcription of the luciferase reporter gene and simultaneously the MYC protooncogene. Both genes are repressed in the presence of doxycycline.

Eight independent tetO-MYC founder lines were established and crossed to the μE-tTA line. The resulting double transgenic mice were then initially analyzed for luciferase expression in different organs (Fig.1*b* and data not shown). The highest luciferase activities were observed in thymus, spleen and lymph nodes. Nonlymphoid organs exhibited only background activity (kidney, brain and muscles) or slightly increased expression (liver) probably because of the presence of lymphoid cells from blood. After doxycycline treatment, luciferase activity was virtually abolished, demonstrating the regulation of tetO-dependent transgenes (data not shown).

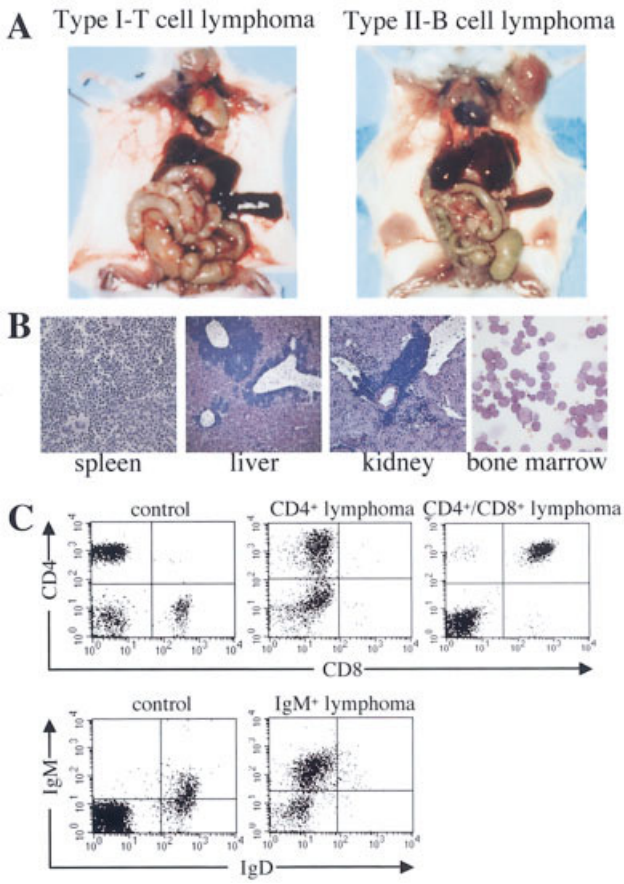

FIGURE 2 – MYC overexpression in lymphoid tissues induces T and B-cell lymphomas. (*a*) tTA/MYC mice with obvious signs of illness were sacrificed and gross anatomy of organs was examined. Two types of morphological changes corresponding to T- (type I) or B- (type II) cell lymphomas were observed. (*b*) Histological examination of organs from mice with advanced lymphomas revealed the presence of lymphoblastic cells in spleen, liver, kidney and bone marrow. (*c*) Tumor phenotypes of T- and B-cell lymphomas corresponding to the above-mentioned type I and II morphologies.

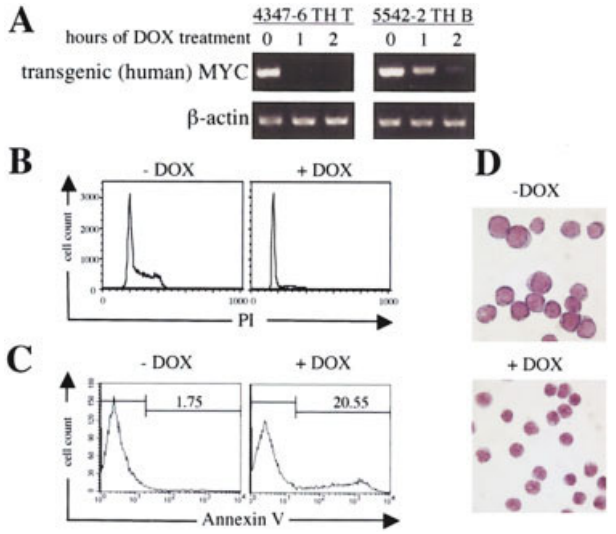

FIGURE 3 – MYC inactivation *in vitro*. (*a*) Tumor cell lines were treated with doxycycline (2 μg/ml) for the indicated times. After treatment, total RNA was isolated, reverse transcribed and PCRs for transgenic (human) MYC were performed. Examples for T- (4347-6TH T) and B- (5542-2TH B) cell lymphomas are presented. (*b–d*) Tumor cell lines were incubated with or without doxycycline for 24 hr and then analysed for various parameters. Cell cycle was analyzed using PI staining (*b*), apoptosis was measured using Annexin V staining (*c*) and cell morphology was determined by Giemsa staining (*d*). Representative data are shown.

Double transgenic tTA/MYC mice were born healthy with the expected Mendelian frequencies. However, all tTA/MYC mice from the line with the highest luciferase activity showed first visible signs of illness at about 10 weeks of age. These mice died within 4 months with an average life span of 13 weeks (Fig.1*c*). Slow movement, ruffled fur, hunched posture and perhaps enlargement of lymph nodes manifested later phases of illness. Most of the experiments were performed with this line. The other 7 tetO-MYC founder lines also exhibited high luciferase expression and they also developed lymphomas, however with somewhat delayed schedule and lower penetrance.

Gross anatomical analyses showed the occurrence of 2 morphologically distinct types of tumors in tTA/MYC mice. Type I was characterized by enlargement of the thymus, spleen, liver and sporadically mesenteric lymph nodes (Fig. 2*a* left). The features of the type II tumors were huge inguinal, cervical and brachial lymph nodes and an increased size of the spleen, the liver and sometimes the thymus (Fig. 2*a* right).

Histological examination of animals with advanced tumors of both types revealed tumor cells within different tissues. Splenic architecture was largely obliterated; no clear distinction between red and white pulp could be made (Fig. 2*b*). Thymus and lymph nodes were also affected (data not shown), while large lymphoblastic cells invaded kidney and liver where they were detected around blood vessels. In addition to the lymphomas, we also detected leukemic cells in bone marrow and blood (Fig. 2*b*,*c*).

To investigate a possible correlation between tumor morphology and tumor cell type, we analyzed leukemic cells from the blood of affected animals for expression of cell surface markers by FACS. Out of 35 analyzed mice, 74.3% developed T-cell lymphomas, whereas the remaining 25.7% developed B-cell lymphomas. T-cell lymphomas corresponded to the type I of gross anatomy. The leukemic cells were surface positive for CD3 and TCR α/β markers. They were either $CD4^+CD8^+$ double positive (84,6%) or $CD4^+$ single positive (15.4%) (Fig.2*c*). B-cell lymphomas represented the type II of gross anatomy. These cells were positive for B220, IgM and CD43, and in some cases showed low levels of CD21 and CD5 (Fig. 2*c* and data not shown). We never observed expression of CD23 and IgD (data not shown), indicating that transformation occurred at an immature stage of B-cell development. Both types of lymphomas were negative for myeloid marker Mac-1 (data not shown).

Establishment of T- and B-tumor cell lines

It was possible to establish appropriate tumor cell lines of both T- and B-cell types of lymphomas. These cell lines allowed us to investigate the consequences of MYC inactivation at the cellular level. Tumor cell lines were first checked for the expression of surface markers and they exhibited the same features as the corresponding primary tumors from donor mice (data not shown). Interestingly, after several weeks of *in vitro* culture all B-cell lines eventually lost expression of surface IgM. Several T- and B-tumor cell lines were checked for expression of human MYC using RT PCR and all of them were positive (Fig. 3*a*). Upon treatment with doxycycline, tumor cell lines aborted expression of human MYC within 1 to 4 hr (Fig. 3*a*). This result is concordant with previously published data indicating that MYC protein and mRNA have short half-lives of about 30 min each.[2] B- and T-tumor cell lines exhibited high luciferase expression, which was reduced to background levels upon doxycycline treatment (data not shown). These results suggested that the activity of the luciferase reporter gene could be used to follow the status of transgenic MYC levels in tumor cells.

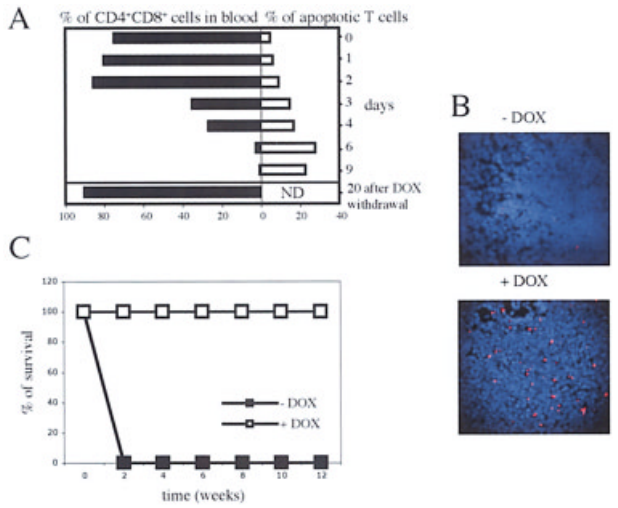

FIGURE 4 – Tumor cells undergo apoptosis upon doxycycline treatment. (*a*) Lymphoma-bearing mice were treated with doxycycline (100 μg/ml in drinking water) for the indicated time. Mice were daily bled before (day 0) and during doxycycline administration and FACS analysis of blood was performed. After 9 days doxycycline treatment was canceled and the mice were bled after an additional 20 days. Representative results showing percentage of tumorigenic ($CD4^+/CD8^+$) and apoptotic (Annexin V^+) cells in gated lymphocytes are presented. (*b*) TUNEL assays revealed apoptosis in the spleen of tumor-bearing mice treated with doxycycline. Slides were counterstained with DAPI. (*c*) 10^7 lymphoma cells were transplanted into syngeneic wild-type recipients. One group of recipients was treated with doxycycline (100 μg/ml in drinking water) immediately upon injection of lymphoma cells. Survival rate of mice (6 per group) is presented.

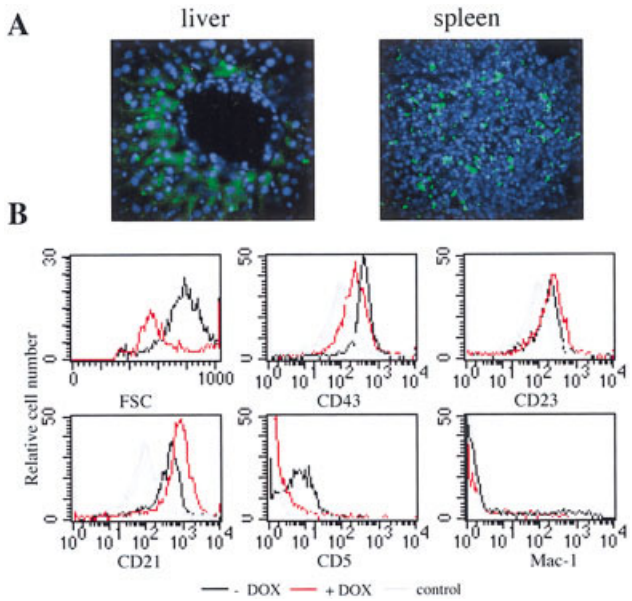

FIGURE 5 – GFP labeled tumor cell lines as a model for examination of the differentiation status Syngeneic recipients were injected with a tumor cell line (10^7 cells), which had been labeled with GFP using retroviral gene transfer. (*a*) After 10 days, mice were sacrificed, sections from liver and spleen were made, stained with DAPI (blue) and analyzed for the presence of GFP^+ cells (green). (*b*) 10 days after transplantation 1 group of recipient mice was doxycycline treated for 5 days (100 μg/ml in the drinking water). Expression of surface markers on isolated GFP^+; cells from treated mice was compared to the expression levels on GFP^+ cells isolated from the untreated group. The gray line represents the staining control (unstained cells in CD5 and Mac-1 staining and Cy-chrome in CD43, CD23 and CD21 staining). Data are representative for 3 mice per group.

Tumor cell lines of both types demonstrated exponential proliferation that was aborted by doxycycline treatment (data not shown). Analysis of DNA content by PI staining confirmed the proliferation arrest upon addition of doxycycline (Fig. 3*b*). Tumor cells treated with doxycycline for 24 hr showed accumulation of cells in the G_0/G_1 phase of the cell cycle. Furthermore, MYC inactivation not only resulted in a proliferative arrest but also in characteristic morphological changes (Fig. 3*d*) and apoptosis (Fig. 3*c*).

Tumor regression upon inactivation of transgenic MYC

We then tested whether suppression of transgenic MYC expression in tTA/MYC mice would result in a reversal of the tumor phenotype. Lymphoma-bearing mice at advanced stages of disease, defined by lymphoadenopathy and presence of lymphoblastic cells in the blood, were treated with doxycycline in the drinking water. Within 2 days of treatment, mice became physically more active and within 1 week showed absence of lymphoadenopathy and lymphoblastic cells in peripheral blood. All tested animals demonstrated initial regression of tumor, while inactivation of MYC was sufficient to induce remission for a prolonged time period in about 70% of cases (Fig. 1*c*). When these tTA/MYC mice were continuously treated with doxycycline, they reached life span of single transgenic control animals. In parallel, we investigated the rate of tumorigenic and apoptotic cells in such doxycycline-treated mice (Fig. 4*a*). Mice positive for the presence of tumor lymphoblastic cells in peripheral blood were chosen. Upon doxycycline treatment, blood was checked for the amount of tumorigenic cells ($CD4_+CD8^+$ double positive cells in the case represented) and cells that undergo apoptosis (Annexin V positive). Our results clearly show that the loss of lymphoblastic cells is associated with increased levels of apoptosis after 3 days of doxycycline treatment. Increased apoptosis correlated with the reduction of the number of neoplastic cells over the following days. Within 9 days, the tumor had entirely regressed and no tumor cells were found in the blood after this time period. However only sustained MYC inactivation reversed the phenotype of tTA/MYC mice. When apparently cured mice were taken off doxycycline, tumors reappeared in these mice within a time period of 2–3 weeks (Fig. 4*a*).

It was shown that Annexin V binds with different affinities to the surface of the lymphocytes at different stages of development.[35] To confirm that the increased proportion of Annexin V positive cells that appeared after doxycycline treatment did indeed represent apoptotic cells, we performed Tunel Assays. Spleen sections of tumor-bearing mice, which were either untreated or treated with doxycycline, were analyzed (Fig. 4*b*). Our results showed high levels of apoptosis after 4 days of treatment with doxycycline. In contrast, only a few apoptotic cells were seen in untreated mice.

To demonstrate that enlarged organs in tTA/MYC mice represent real tumors rather than a massive proliferation of activated cells, we transplanted several of the tumors to healthy syngeneic mice. Single cell suspensions were made from primary tumor tissues and either injected directly into recipient mice or they were previously cultured *in vitro*. Within 2 weeks mice injected with T- or B-cell lymphomas developed tumors, which expressed the same cell surface markers as the donor tumors (data not shown). Syngeneic mice with transplanted tumors recapitulated the pattern of tumor invasion of primary transgenic tumors (data not shown). Similarly to the results obtained with the original MYC transgenic mice, transplanted tumors rapidly regressed upon doxycycline treatment, while nontreated animals died as a consequence of aggressive lymphomas (Fig. 4*c*).

Differentiation as a mechanism of tumor elimination

Previously described data suggested that apoptosis and differentiation are the main mechanisms responsible for tumor elimina-

tion in MYC transgenic mice.[20] In order to investigate whether differentiation occurs as a consequence of doxycycline treatment, we used GFP to mark the tumor cells. Retroviral gene transfer was used to express the GFP protein in some of the tumor B-cell lines. Such GFP marked cells were then injected into syngeneic mice. This approach allowed us to distinguish neoplastic cells from normal recipient's lymphocytes. Recipients again developed aggressive lymphomas that invaded different lymphoid and nonlymphoid organs. We were able to detect GFP positive cells in histological sections from spleen and liver (Fig. 5*a*) but also by FACS analysis in blood, lymph nodes and spleen (data not shown). Analysis of the surface marker expression profile revealed that the GFP-labeled tumor cell line expressed significant amounts of CD21 and CD43, while the level of CD23 was low (Fig. 5*b*). As already mentioned, during the *in vitro* cultivation period the B cells lost expression of surface IgM. This IgM expression did not reappear in the transplanted tumors. CD5 was expressed on the surface of these cells; however we could not detect presence of Mac-1, indicating that these cells were not canonical B1 B cells (Fig. 5*b*). The identical patterns of cell surface marker expression were detected on cells before and after GFP labeling as well as after injection into the syngeneic mice (data not shown and Fig. 5*b*). After tumors had developed in recipient mice, 1 group of mice was treated with doxycycline, and 5 days later, the GFP positive cells were analyzed and compared to those from untreated animals. In line with earlier data,[20] doxycycline treatment led to a reduction in the size of tumor cells (Fig. 5*b*). Interestingly however, surface marker expression also changed upon treatment. We observed a reduction of CD43 as well as of CD5 expression levels (Fig. 5*b*), indicating that downregulation of transgenic MYC could indeed induce differentiation. However, while expression of CD21 was slightly increased, the level of CD23 was not significantly altered by MYC inactivation and we were not able to detect IgM or IgD on the cell surface. Thus these cells exhibit only a partial progression along the B-cell differentiation pathway.

Genome instability in tumor cell lines

Several *in vitro* analyses had shown that MYC activation induces the rapid accumulation of chromosomal abnormalities.[26–28] We therefore asked whether MYC was able to induce genomic instability in our *in vivo* model of lymphomagenesis. To answer this question, we performed CGH (Comparative Genomic Hybridization) analysis. DNAs from different T- and B-lymphoma cell lines and normal reference DNA were isolated, labeled and hybridized to normal denatured metaphase spreads. In all analyzed tumor samples, chromosomal aberrations in the form of loss or gain of chromosomal material were detected (data summarized in Table I). The most common finding was a gain of chromosome 15 in T-cell tumors. In 4 out of 7 independently established cell lines from different animals that had developed T-cell lymphoma, this gain of chromosome 15 sequences was observed. The gain of chromosome 15 was present in all cell lines made from the same animal, no matter from which organ the line was derived and no matter how many passages the cells were cultured [from mouse 4347-6 lines: 4347-6SP (T), 4347-6TH (T)-a and 4347-6TH (T)-b and from mouse 7755-4 lines: 7755-4TH (T)-a and 7755-4 TH (T)-b]. The gain of chromosome 15 was not observed in any of the B-cell lymphomas analyzed. In the case of 2 cell lines [4347-6TH (T) and 7755-4TH (T)] that were analyzed for the second time after increased number of passages, additional chromosomal alterations were found.

A whole chromosome paint (WCP) of chromosome 15 showed that the detected gains of chromosome 15 actually represented trisomy of this chromosome (Fig. 6*a*). All 3 copies of chromosome 15 seem to be normal as no gross structural differences were detectable. This result was further confirmed by 2-color FISH hybridizations with different combinations of BAC probes specific for genes, which are all localized at 62.4–103.9 Mb on chromosome 15 such as MYC, Tgn, Pdgf2, Ly6, Itgb7 and the Hox gene cluster (data not shown).

TABLE I – CGH ANALYSIS OF TUMOR CELL LINES[1]

Cell line	Imbalances	
	Gains	Losses
1415-2TH (T)	1B-H	
1749-2SP (T)	3; 15	
3445-2TH (T)	11A4-E; 14A-C	
4347-6SP (T)	15	
4347-6TH (T)-a	15	
4347-6TH (T)-b	3D-F; 15	
4491-8TH (T)	10; 15	1C3-D
4502-8TH (T)	12A-C2	
5532-2SP (B)		1C5-D; 3A2-E2; 14C
5532-2TH (B)		3A2-F1
5534-2SP (B)	10	
7755-4TH (T)-a	15	
7755-4TH (T)-b	10B-D; 15	

[1]Several T- and B-cell lymphoma lines, (T) or (B), were analyzed for chromosomal instabilities. Tumor cell lines 4347-6SP (T) and 4347-6TH (T), and lines 5532-2SP (B) and 5532-2TH (B) were derived from the same mouse respectively (mouse 4347 and mouse 5532) but were established from different organs (SP-spleen, TH-thymus). Also 2 lines were analyzed for a 2 time after additional number of passages (-a for the first analysis and -b for the second analysis).

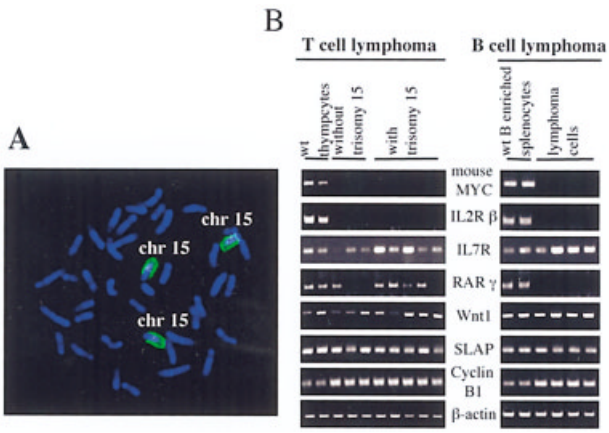

FIGURE 6 – MYC overexpression leads to genomic instability. (*a*) Cell lines with the observed gain of chromosomal material corresponding to chromosome 15 by CGH analysis were subjected to fluorescence *in situ* hybridization (FISH) using whole chromosome 15 probes. The representative result shows chromosome 15 trisomy in 1 of the analyzed tumor cell lines. (*b*) RNA was isolated from T-cell lines with or without chromosome 15 trisomy, as well as B-cell lines. RT-PCR analysis of the indicated genes was performed. As a control wild-type thymocytes or B-cell-enriched splenocytes were used. Primers specific for β-actin were used as control.

Expression of chromosome 15 related genes

To determine whether trisomy of chromosome 15 leads to increased expression of endogenous (mouse) MYC, we compared the expression levels of MYC mRNA in the samples of the T-cell lines with and without chromosome 15 trisomy as well as in the B-cell lines. As a control, mRNA from wild-type mouse thymocytes or splenic B cells was used. While in the control samples mouse MYC mRNA was clearly present, we were not able to detect murine MYC mRNA in any of the tested tumor cell lines made from tTA/MYC mice (Fig. 6*b*). This result is in agreement with previously published data showing that transgenic MYC results in repression of the endogenous MYC gene.[19]

In addition to the MYC gene, chromosome 15 contains several additional genes with known functions in lymphocyte proliferation and/or tumorigenesis. We analyzed mRNA levels of IL-2 receptor

beta (IL-2Rβ), IL-7 receptor, retinoic acid receptor gamma (RAR γ), Wnt 1, SLAP and cyclin B1 in order to investigate whether expression of these molecules was altered thus contributing to the MYC-induced lymphomagenesis. Compared to control cells, all tumor cell lines expressed increased levels of cyclin B1. Furthermore IL-7R mRNA expression was high in all B-cell lines and some of the T-cell lines (Fig. 6*b*). No obvious difference was observed in the case of SLAP and Wnt 1 genes (Fig. 6*b*). Intriguingly, expression of mRNA for IL-2 receptor beta was completely blocked in tumor cells (Fig. 6*b*). Since IL-2Rβ signaling was shown to regulate expression of MYC,[36,37] this result suggests that reduction of IL-2Rβ expression could be part of the feedback mechanism by which cells regulate MYC protein levels. Interestingly, while expression of virtually all of these genes differed between wild-type and tumor cells, we did not observe any significant difference between tumor cell lines with and without chromosome 15 trisomy.

DISCUSSION

Association of MYC expression with tumorigenesis in various tissues is consistent with the crucial role for this oncogene in the regulation of cell growth and development. Different MYC transgenic models established in the past confirmed this hypothesis. It has been shown that elevated MYC expression could result in a variety of tumors including testicular tumors, β cell tumors in the pancreas, papillomatosis and mammary adenocarcinomas.[21,38–40] Many efforts have been made to develop mouse model of MYC-induced leukemias. While some of these models used promoters expressed in wide variety of tissues,[38] others were based on the Eμ enhancer similar to our study. Felsher and Bishop employed a very similar strategy to control MYC expression in lymphoid tissues.[20] While in most Eμ-MYC based models, elevated expression of MYC led to the development of B-cell lymphomas, they found predominantly T-cell lymphomas and some myeloid leukemias.[20] In contrast, our mice developed both T- and B-cell lymphomas although the latter to a lower extent. The expression cassette used to drive tTA in our mice had been studied earlier and it had been shown to function efficiently in bone marrow, thymus, spleen and lymph nodes.[34] The fact that the highest luciferase activities were observed in the thymus might explain the prevalence of T-cell lymphomas in our study.

We could show that lymphomas that developed in our model were transplantable, thus proving that accumulation of lymphocytes in the lymphoid organs and the blood of tTA/MYC mice was not due to an increased proliferation of the reactive cells but indeed due to MYC-induced tumorigenesis. Furthermore, cells isolated from transgenic mice could be easily adapted to the growth in cell culture without any specific growth factors.

Gross anatomy of affected mice demonstrated a dual origin of developed lymphomas with characteristic histological features of the B- and T-cell lymphomas, respectively. Tumor cells exhibited hallmarks of early stages of lymphocyte development. T-cell lymphomas were mostly CD4/CD8 double positive with only 15% of them being CD4 single positive. The B-cell lymphomas we observed resembled those described in a mouse model of Burkitt lymphoma.[22] They expressed IgM and CD43 and very low levels of CD23. However, opposite to Burkitt lymphoma cell lines, our B-cell lymphomas lost surface IgM after *in vitro* cultivation. This phenotypic instability could suggest that in our mice MYC-induced transformation had occurred at an earlier stage of B-cell development.

Treatment of tumor-bearing mice or established cell lines with doxycycline demonstrated that all lymphomas were dependent on continuous MYC expression. In the presence of doxycycline, cells rapidly lose expression of transgenic MYC protein and this results in growth arrest at the G_1 stage of cell cycle and induction of apoptosis. Tumor regression and apoptosis were also readily apparent in mice after MYC inactivation. The correlation between increased apoptosis and reduction of tumor cell numbers indicates that apoptosis is a major mechanism of tumor elimination. Furthermore, Felsher and Bishop[20] had interpreted morphological changes of tumor cells upon MYC inactivation as evidence for cellular differentiation. Our data also strengthen this latter hypothesis. The GFP-labeled B-cell lymphoma cells used in our study did not reach the stage of terminal maturation. It is well documented that development of B cells depends on BCR signaling.[41] Therefore, the observed loss of surface IgM during *in vitro* cultivation of B cell most likely limited their ability to accomplish terminal differentiation. Nevertheless, the alterations in CD43 and CD21 surface expression levels indicate that the cells proceeded at least partially in their differentiation upon MYC inactivation.

Induction of genomic instability by MYC overexpression was already postulated according to the data obtained in different *in vitro* models. It was described that MYC can induce genomic instability *in vitro* in IL-3 dependent murine Ba/F3 cells[27] and in the Rat1A cell line defective for the ARF/MDM2/p53 pathway.[26] The same effect of MYC was also shown in cell culture of normal human fibroblasts.[28] Similar findings were obtained in *ex vivo* analyses of MYC transgenic mice on a p53-deficient background.[42] In line with this, our data show the ability of MYC alone to induce genomic instability *in vivo*. Interestingly, it seems that genomic instability induced by MYC are not randomly distributed throughout the genome. We could observe that in several cell lines chromosomes 1, 3, 10 and 14 were affected. Abnormalities of chromosomes 1 and 14 as a consequence of MYC action were already described in BALB/c mice prone to development of plasmacytomas.[43] Importantly, we found that more than 50% of the T-cell lymphomas analyzed have an additional copy of chromosome 15. The gain of chromosomal material that corresponds to chromosome 15 has been frequently observed in different mouse tumor models.[44] Often, this gain was due to amplification of MYC itself and has been considered as directly responsible for tumor development.[44–46] We observed a complete trisomy of chromosome 15 but no increased copy number of the endogenous MYC gene or any of other analyzed genes. The presence of an additional copy of chromosome 15 in tTA/MYC mice in fact does not lead to increased amounts of mouse MYC mRNA. In contrast, we were not able to detect mRNA encoding endogenous MYC in any of analyzed cell lines. This finding confirms earlier results that the MYC gene is subjected to negative feedback control by an excess of transgenic MYC.[19,47,48]

We also analyzed mRNA expression of several others genes located at chromosome 15 that might have a role in the regulation of cell proliferation and differentiation as well as in tumorigenesis. Although we observed differences between tumor and wild-type cells, no consistent differences in expression of those genes were observed between the samples of tumor cells with and without chromosome 15 trisomy.

The significance of genomic instability in MYC-induced tumorigenesis still is not resolved. Different authors suggest genomic instability as a crucial step in tumorigenesis.[26] In contrast, others consider it as a side-step and consequence of neoplastic process.[28] Complete dependence of developed tumors in tTA/MYC mice on sustained MYC activation could argue in favor of the latter opinion. However, previous studies described MYC induced tumors as monoclonal.[18,20] Our findings that we never observed different lymphoma types in 1 mouse and the observation that same types of genomic instabilities were detected in tumor cells isolated from different organs of 1 mouse also imply tumor monoclonality. Additionally, we did not observe any alteration in size and cellularity of lymphoid organs in preneoplastic mice. Together, these data suggest that although tumors were MYC dependent, additional events are clearly necessary for MYC-induced neoplastic transformation. Although genomic instability seems to be important element of MYC-triggered tumorigenesis, we do not know the exact nature of these further events.

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