Deregulated expression of Myc under the control of an immunoglobulin enhancer induces lymphoma formation in mice. The development of lymphomas is limited by TGFβ-dependent senescence and high levels of Myc expression are continuously required to antagonize senescence. The biological processes underlying senescence are not fully resolved. We report here a comprehensive analysis of TGFβ-dependent alterations in gene expression when the Myc transgene is switched off. Our data show that Myc-induced target genes are downregulated in a TGFβ-independent manner. In contrast, TGFβ is required to upregulate a broad spectrum of genes that are characteristic for different T-cell lineages when Myc is turned off. The analysis reveals a significant overlap between these Myc-repressed genes with genes that are targets of polycomb repressive complexes in embryonic stem cells. Therefore, TGFβ-dependent senescence is associated with gene expression patterns indicative of abortive cellular differentiation along several lineages.

Introduction

A current paradigm of molecular oncology holds that mutational activation of single oncogenes is insufficient to cause tumors since such mutations also trigger failsafe mechanisms such as apoptosis and senescence.1,2 Tumorigenesis requires that failsafe mechanisms are compromised or inactivated by secondary mutations. One experimental system, in which this paradigm has been extensively studied is Myc-induced lymphomagenesis in Eμ-Myc transgenic mice. These mice express Myc under the control of the immunoglobulin heavy chain enhancer, modeling the chromosomal translocations found in human Burkitt's lymphoma and mouse plasmacytoma.3 A variation of this model are bi-transgenic mice, that express a tetracycline- (doxycycline-) regulated transactivator protein (tTA) under the control of the Eμ enhancer and a Myc-transgene under a tTA-responsive promoter, allowing inducible and reversible expression of Myc.4 In these mice, reversal of Myc expression leads to rapid tumor regression of established lymphomas, providing evidence that lymphomas are “addicted” to high levels of Myc.

Myc-induced lymphomagenesis is limited by apoptosis. A number of molecular processes underlying this failsafe mechanism have been resolved.3 More recently, senescence has emerged as a further mechanism that limits Myc-induced lymphoma formation. This notion is supported by three lines of evidence: first, apoptotic B-cell lymphoma cells attract macrophages that secrete TGFβ and induce senescence in a certain percentage of cells even in the presence of deregulated Myc.5 The precise mechanism underlying this response is unknown, but the histone H3K9 methyltransferase, SuVar39 is required for induction of senescence.6 Second, lymphomas generated by a mutant allele of
Myc, MycV394D, arise more slowly. The delay in tumor formation in mice harboring the mutant allele is reflected by an increased percentage of senescent lymphoma cells. The encoded gene product fails to repress transcription via complex formation with Miz1; senescence correlates with impaired cell cycle progression and enhanced expression of two cell cycle inhibitors, cdkn2b (encoding p16ink4a) and cdkn1c (p57Kip2), which are induced by TGFβ and repressed by wild type Myc via its interaction with Miz1. The MycV394D mutant is defective in Miz1 binding and transcriptional repression, indicating that these activities of WT Myc are critical to prevent Myc-induced senescence. For example, deletion of Cdk2 induces cells expressing high levels of Myc to undergo senescence, whereas normal somatic cells do not depend on Cdk2 for proliferation.

Upon reversal of the expression of the c-myc transgene, Myc-induced T-cell lymphomas undergo G1 arrest and apoptosis, whereas the ensuing genome-wide profile of tumor regression. This analysis identified large groups of genes that were up and downregulated when expression of the c-myc transgene was reversed, confirming previous reports that Myc can control the expression of large sets of genes (Fig. 1A). Regulation of a subset of both down and upregulated genes depended on TGFβ, since their regulation was altered in cells expressing TβR-II-ED. We have previously demonstrated that the cell cycle inhibitors, cdkn2b and cdkn1c, are induced in a TGFβ-dependent manner under these circumstances.

To identify whether a defined functional class (GO-terms) is enriched within this group of genes we analyzed them with the functional annotation module of the Database for Annotation, Visualization and Integrated Discovery (DAVID). Additionally we analyzed the expression profile for the enrichment of results from previously identified expression profiles using gene set enrichment analysis (GSEA). To discriminate between genes directly and indirectly regulated by Myc, we used a genome-wide Myc-binding map generated by ChIP-sequencing analysis in Eu-Myc mice at the pre-tumoral and tumoral stages and overlapped these binding data with our expression analysis (Fig. 2).

The set of genes that were downregulated in response to de-induction of the c-myc transgene was highly enriched for direct target genes of Myc (Fig. 2A). Consistent with current concepts of Myc function, many of these genes encoded proteins involved in protein biosynthesis, translation and cell cycle progression (not shown). These data support a straightforward model, in which direct binding of Myc to the promoters of these genes enhances their expression. Consistent with such a model, the vast majority of these genes were repressed in a TGFβ-independent manner when the c-myc transgene was switched off (858/922 genes). In contrast, the analysis of the small group of genes that was repressed in a TGFβ-dependent manner did not yield a significant enrichment of functional gene classes (by GO-term analysis).

About 25% of the genes that were upregulated in response to de-induction of c-myc depended on TGFβ, paralleling induction of cellular senescence. Surprisingly, GO-term analysis revealed that these genes were highly enriched for genes involved in epidermal, skin and muscle development (Fig. 1B). This finding was confirmed by a GSEA analysis, which revealed highly significant overlaps with gene sets characteristic for ectoderm, mammary, skeletal and muscle development (Fig. 1C). Notably, the most significant overlap was found with a set of genes that includes targets of the transcription factor MyoD, a master regulator of muscle differentiation. In contrast, the set of genes that was upregulated in a TGFβ-independent manner did not show any enrichment for genes involved in developmental decisions (not shown). Rather these genes were enriched for genes controlling lymphocyte activation, signal transduction and apoptosis, potentially reflecting the TGFβ-independent apoptosis that takes place in these cells when the c-myc transgene is switched off.

Taken together, the data show that TGFβ-dependent induction of senescence upon de-induction of c-myc closely correlates with expression of a large set of genes that are characteristically expressed in several different T-cellular lineages. The data establish a relation between the induction of senescence and a—likely abortive—differentiation of lymphoma cells when the c-myc transgene is turned off. Previous work had suggested that high levels of Myc enforce a gene expression profile that is characteristic of embryonic stem cells. In such cells, tissue specific genes such as those described above are repressed, since they are direct or indirect target of the polycomb family of repressor proteins. We therefore asked whether the class of genes that is upregulated in a TGFβ-independent manner when c-myc expression is reversed shows enrichment for genes that are bound by polycomb in embryonic stem cells (Fig. 2B). Consistent with this suggestion, GSEA analysis revealed a highly significant overlap between both groups of genes, arguing that repression of polycomb target genes is one mechanism by which high levels of Myc prevent TGFβ-dependent senescence in T-cell lymphomas. Comparison with the set of genes directly bound by Myc in B-cell lymphomas showed that only a minority
of genes that correlated with induction of senescence were direct target genes of Myc (Fig. 2A). Importantly, this was also true for the set of genes that are bound by polycomb proteins in ES cells, demonstrating that Myc-mediated repression of these genes occurs indirectly (not shown).

Discussion

We report here a comprehensive analysis of changes in gene expression that correlate with induction of senescence in a mouse model of Myc-driven lymphomagenesis. Lymphomas that arise in this model are addicted to high levels of Myc, since de-induction of the Myc transgene leads to rapid tumor regression. Our previous work has shown that lymphoma cells secrete high levels of TGFβ-2 and -3 and that high levels of Myc are continuously required to suppress TGFβ-dependent
for example. The findings show that Myc-driven lymphoma cells are able to express genes of different lineages when Myc-expression is reversed. Previous work had established that high levels of Myc can enforce gene expression patterns that are characteristic for embryonic stem cells. Furthermore, reversion of transgene expression in a Myc-driven model of hepatocellular carcinoma induces differentiation of tumor cells into hepatocytes and biliary cells, documenting that tumor cells are pluripotent. Our data confirm and provide a striking example for the degree of pluripotency, since, upon de-induction of c-myc, lymphoma cells express marker genes that are not characteristic for hematopoietic lineages.

Our data also raise issues about the molecular processes that underlie senescence in this and related biological models. Notably, polycomb-mediated repression of the cdkn2a locus, which encodes both p16Ink4a and p19Arf, is a critical mechanism that prevents senescence. Ectopic expression of Myc does not support polycomb-mediated repression of both genes, since high levels of Myc induce p19Arf and also p16Ink4a expression. However, the data presented here document a high degree of overlap between genes encoding developmental regulatory proteins repressed by Myc and target genes for polycomb proteins in embryonic stem cells. Taken together, the data suggest that senescence may be causally linked to—likely abortive—differentiation along several developmental lineages.

Finally, we have not clarified the mechanisms by which Myc acts to repress polycomb target genes since only a small subset of these genes is bound directly by Myc in B-cell lymphomas. While it is possible that Myc occupies different loci in T and in B-cell lymphomas, it appears more likely that repression is indirect, potentially involving regulation by Myc of genes encoding members of the polycomb repressor complex.

Materials and Methods

Animal experiments. The generation of transgenic mouse lines for conditional expression of MYC (TetO-MYC) has been described in reference 20.

Figure 2. TGFβ-dependent gene expression profile correlates with polycomb target genes. (A) Pie charts documenting the number of genes directly bound by Myc in B-cell lymphomas among the different categories shown in (A) of Figure 1. (B) GSEA analysis documents that genes that are up-regulated in the absence of Myc in a TGFβ-dependent manner significantly overlap with targets of the polycomb repressor complex. Each probe belonging to the polycomb gene set is visualized as a vertical line below the X-axis. The green curve signifies plotting of the enrichment scores with a maximal negative enrichment score of -0.35. The statistical analyses are listed below.
Lymphoma-derived cells were infected with either TβR-II-ED or empty vector control (pMSCV-GFP) as described before. For transplantation experiments 10 x 10^6 cells were washed once in PBS before subcutaneous (s.c.) injection into FVB/N syngeneic mice. To turn off MYC transgene expression, drinking water of mice was supplemented with 200 µg/ml doxycycline. All animal experiments were performed in accordance with the guidelines from Administrative Panel on Laboratory Animal Care at Stanford University (protocol 14045).

**Microarrays and analysis.** Total RNA was isolated from mouse tumor tissue using the RNeasy Kit including DNase-I digest (Qiagen, Valencia, USA) following the manufacturer’s protocol. Microarrays were scanned on the Agilent scanner according to the manufacturer’s protocol. Microarray image files were analyzed using Agilent’s Feature Extraction software v. 7.5. To account for spot differences, the background-corrected ratio of the two channels was calculated and log2 transformed. To balance the fluorescence intensities for Cy3 and Cy5 as well as to allow for comparison of expression levels across experiments, the raw data were standardized. We used the loess normalization to correct for inherent bias on each chip. To find differently expressed genes, changes in mRNA expression levels were calculated for each gene and genes showing at least a twofold change were chosen as differentially expressed.

Changes in the expression of functionally related genes were analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) and the Gene Set Enrichment Analysis (GSEA, version 2, Broad Institute, Cambridge, MA). DAVID was used on the web site with the predefined settings. The data have the ArrayExpress accession number: E-MEXP-2233.

For the GSEA analysis, genes represented by more than one probe were collapsed to the probe with the maximum value using the gene symbols. Genes were ranked based on the mean log2 Ratio. A false discovery rate (FDR) ≤ 25% was used as parameter to detect differentially regulated gene sets.

Expression data and gene annotations were stored in Array Express, which complies with MIAME (minimal information about a microarray experiment) guidelines. The R environment software http://www.r-project.org was used for data analysis.

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**References**