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Functional Validation of Genes Implicated in Lymphomagenesis:

An *in Vivo* Selection Assay Using a Myc-Induced B-Cell Tumor

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Abstract

The involvement of the c-Myc transcription factor in neoplastic transformation is well documented. However, which of its numerous target genes are crucial for tumorigenesis remains a frequently contested issue. We have recently established a non-transgenic murine model for B-cell lymphoma based on neoplastic conversion of p53-null bone marrow cells by conditionally active Myc. Using this model, we have identified a number of genes whose expression levels are affected by Myc during B-lymphomagenesis. Here we discuss their possible roles in neoplastic processes and describe an experimental approach allowing *in vivo* validation of these roles. We demonstrate that lymphoma cells overexpressing one of the Myc targets, the interleukin-10 receptor gene, have a very strong selective advantage over low IL10R expressors. Furthermore, Mcl1, a presumptive IL10R effector, also confers selective advantages when overexpressed in Myc-transformed hematopoietic cells. Thus, both IL10R and Mcl1 might be amenable to therapeutic interventions, and new targets can be identified and validated using the selection approach.

Keywords

c-Myc; B-cell lymphoma; target genes; Mcl1; IL10

INTRODUCTION

c-Myc, an oncogenic transcription factor, is a crucial regulator of cell expansion. It was realized early on that activation of Myc forces quiescent fibroblasts to re-enter the cell cycle¹ and that rodent fibroblasts with targeted disruption of Myc are severely deficient in cell proliferation.² Thus, in mice (but curiously not in *Drosophila*³), decreased expression of Myc results in hypoplasia.⁴ Predictably, some of the best-recognized Myc targets are crucial regulators of cell homeostasis: cdk4⁵ and telomerase⁶ (upregulation) and cdk inhibitors⁷⁻¹¹ and gadd45¹² (downregulation). In addition, Myc regulates, at the transcriptional level, a dazzling array of genes pertaining to numerous physiological and pathological processes, such as cell growth and cell death.^{13,14} In all, the database of Myc-regulated genes currently contains 1,697 entries (<http://www.mycancergene.org/>). It is often pointed out that many of these genes are indirect targets and that only a fraction of them might contain *bona fide* Myc-binding sites. Yet recent research using chromatin immunoprecipitation and other approaches has revealed that Myc binds to and regulates up to 15% of all genes (reviewed in Ref. 15). The abundance of Myc target genes raises the question of how crucial mediators of Myc-induced tumorigenesis could be identified.

Below we describe a simple approach that has allowed us to assign functional significance to Myc-induced variations in interleukin-10 receptor levels in transformed bone marrow cells.

According to our data, the *il10ra* gene is downregulated by Myc,¹⁶ and its expression levels become rate-limiting for B-lymphomagenesis. However, in the absence of active Myc, IL10R protein levels rapidly recover. This boosts cell expansion and ultimately correlates with Myc-independent tumor relapse. We envision that the same approach could be used to functionally validate various Myc targets as well as other genes tentatively implicated in B-lymphomagenesis.

MATERIALS AND METHODS

Tumor Production and Analysis

Generation of Myc- and MycER-induced murine B-lymphomas has been described earlier.¹⁶⁻¹⁸ Syngeneic C57BL/6/J mice (NCI, Frederick, MD) were used as hosts for tumor production. Lymphoma cells ($5-10 \times 10^6$) were injected subcutaneously into the flanks, and tumor growth was monitored over the course of several weeks. For 4-OHT treatment, the hormone powder (H6278, Sigma, St. Louis, MO) was dispersed in corn oil (Sigma) at the concentration of 10 mg/mL. The administration of 4-OHT was performed daily via intraperitoneal injections (1 mg per mouse). For the selection assay, tumors were harvested and dissected approximately 3 weeks after implantation. Neoplastic cells were used for flow cytometric analyses or subsequent passages *in vivo*.

Microarray Analysis

Total RNAs from MycER tumors were used for cDNA synthesis. Labeled probes were hybridized to the U74v2 gene chip (Affymetrix, Inc. Santa Clara, CA) using the PENN Microarray Facility's standard protocol (<http://www.med.upenn.edu/microarr/Data%20Analysis/Affymetrix/methods.htm>). Affymetrix MAS 5 probeset signals and presence/absence flags were calculated. The LPE (Local Pooled Error) test for differential expression as implemented in S+ArrayAnalyzer v 1.1 (Insightful Corporation, Seattle, WA) was applied with 1% Bonferroni multiple testing correction to median IQR normalized MAS 5 signal values. The resulting list of 686 genes was imported into GeneSpring v 6.1 (Silicon Genetics, San Carlos, CA), filtered for presence (per Affymetrix MAS5 analysis) in 2 of 2 samples in one or more conditions (Myc^{ON} or Myc^{OFF}), and then filtered for fold change.

Generation of Recombinant Retroviruses

The murine IL10R α -encoding retrovirus has been described earlier.¹⁶ To generate an IL10R α mutant lacking the intracellular domain, the PCR α -mIL10R α plasmid¹⁶ was cut with BamH I and Xma I, and the truncated IL10R insert was subcloned into the pSL1180 phagemid vector (Amersham Pharmacia, Piscataway, NJ). In this vector, the polylinker segment between Xma I and Sal I site was replaced with the double-stranded oligonucleotide (CCGGGTAGAAATTACCTCAG) containing a single T-to-G substitution shown in bold. This substitution resulted in a stop-codon (underlined) positioned in-frame with the IL10R coding sequence. The IL10R_{STOP} sequence was cut out with Bgl II and Sal I and inserted into the bicistronic GFP-expressing retroviral vector pMIGR1¹⁹ linearized with Bgl II and Xho I. The full length murine mcl-1 cDNA was purchased from the American Type Culture Collection (Manassas, VA, catalog # MGC-13884, IMAGE ID: 4190996). The cDNA was released from the pCMV-SPORT6 vector using Not I and EcoR V restriction enzymes, cloned into pSL1180, and then recloned, using Bgl II and Hpa I restriction sites, into MIGR1.

Cell Propagation, Transfection, and Infection

Myc3 B-lymphoma cells were cultured on monolayers of gamma-irradiated S17 cells as described previously.¹⁸ BOSC and GP293 packaging cells were cultured in DMEM with 10% FBS. For *in vitro* infections, either of these cell lines was transfected with retroviral clones

using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Conditioned media were harvested and added to Myc3 cells for 2 to 12 h. Three days post infection, the percentages of GFP-positive cells in infected cultures were determined using flow cytometry.

Flow Cytometric Analyses

To detect expression of mIL10R α and the mIL10R_{STOP} truncated protein, transiently transfected packaging cells were stained on ice for 45 min with a biotin-conjugated anti-mouse IL10R antibody (559913, Pharmingen, San Diego, CA), followed by staining with APC-conjugated streptavidin (SA1005, Caltag Laboratories, Burlingame, CA). All samples were analyzed using a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA) with the CELLQuest software (Becton Dickinson).

RESULTS AND DISCUSSION

Improved Survival of Neoplastic Cells following Myc Inactivation

We have recently established a new mouse model for B-cell lymphoma based on infection of p53-null bone marrow cells with retroviruses encoding either constitutively active Myc¹⁷ or a fusion between Myc and the mutant form of the estrogen receptor (MycER),²⁰ requiring the presence of the synthetic estrogen 4-hydroxytamoxifen (4-OHT) for activity.¹⁶ The use of conditionally active Myc allowed us to address the role of Myc in tumor maintenance (Fig. 1). To this end, cells obtained from primary tumors were injected subcutaneously into flanks of syngeneic mice, and administration of the estrogen was maintained until palpable neoplasms have formed. Animals were then randomized into three groups. Mice in the first group continued to receive 4-OHT and rapidly developed large tumors (Fig. 2, tumors 1 and 2). In the second group, 4-OHT treatment was permanently discontinued (tumors 3 and 4). In the third group, 4-OHT treatment was suspended for two weeks and resumed on day 21 (tumors 5 and 6). We observed that in animals deprived of 4-OHT, tumor growth decreased dramatically, but the neoplasms did not undergo overt regression. Instead tumors remained stable for approximately two weeks. If 4-OHT was re-administered, extremely rapid neoplastic growth ensued (tumors 5 and 6), attesting to the viability of cells with inactivated Myc. Moreover, even without estrogen re-administration, MycER tumors began to slowly regrow after only two weeks of dormancy (Fig. 2 and Ref. 16).

To correlate tumor dormancy with neoplastic cell survival, hematoxylin/eosin and TUNEL stainings of formalin-fixed tumor sections were performed. We observed that while the rate of cell proliferation decreased upon inactivation of Myc (data not shown), so did the rate of cell death. Interestingly, tumors with reactivated Myc exhibited yet higher rates of apoptosis, as judged by the percentage of TUNEL-positive cells (Fig. 2). Conversely, relapsing tumors with inactivated MycER had modest rates of cell proliferation but almost no apoptosis.

Transcriptional Signatures of Myc^{ON} and Myc^{OFF} Tumors

In order to correlate Myc-induced changes in cell proliferation and survival to alterations in gene expression, we performed a series of microarray experiments. In these experiments transcriptional signatures of tumors with active and inactive MycER were compared. Differentially expressed genes were identified as described in MATERIALS AND METHODS. Forty-four genes were upregulated more than fourfold in Myc^{ON} cells and 68 genes in Myc^{OFF} cells (TABLES 1 and 2).

The functions of many Myc-upregulated and Myc-downregulated genes were consistent with the biological findings. Several genes implicated in cell proliferation maintained high transcription levels only in the *presence* of functional MycER, for example, cyclins B1 and B2, cdc2A, and DP1 (Table 1). On the other hand, many of the genes upregulated in the

absence of Myc were known to contribute to B-cell survival and expansion, in particular, in response to antigen or cytokine stimulation. This list included components of B-cell receptor signaling pathways (receptor type protein tyrosine phosphatase C and B-lymphoid kinase),²¹ NF- κ B-activators (lymphotoxin B),²² direct apoptosis inhibitors (Mcl1),²³ and cytokine receptors (interleukin 10 receptor)²⁴ (Table 2 and data not shown). While their expression correlated with neoplastic B-cell survival and eventual relapse, some of them were incompletely characterized or even reported to suppress tumorigenesis. For example, while IL10 has been known to stimulate proliferation of normal B cells *in vitro*,²⁵ its *in vivo* effects on both normal and neoplastic B cells are not well understood. In addition, anti-angiogenic, and thus anti-neoplastic, effects of IL10 have been reported.²⁶⁻²⁸ Thus, we were interested in developing an unbiased approach capable of revealing both tumor-promoting and tumor-inhibiting properties of IL10R and other Myc target proteins.

An *in Vivo* Selection Assay for Cells Overexpressing Myc Target Genes

The following approach was devised in order to assign functional significance to genes implicated in B-lymphomagenesis, such as IL10R. Its full-length cDNA was inserted into a retroviral vector MIGR1, co-expressing through the IRES element green fluorescent protein (GFP).¹⁹ Retroviral constructs were transfected into packaging cells, and viral supernatants were harvested 48 h later. Then Myc-overexpressing B-lymphoma cells (Myc318) were infected *in vitro* with either MIGR1/IL10R or just MIGR1 (empty vector). By varying the amount of viral supernatants, the infection rate of approximately 10% was achieved, as judged by flow cytometry for GFP. Partially but stably infected cultures were used for tumor production in syngeneic animals. The resulting tumors were analyzed using flow cytometry and percentages of GFP-positive cells were determined again. By enumerating GFP-positive cells before and after passaging *in vivo*, both positive and negative effects of the gene of interest on B-lymphomagenesis could be revealed (Fig. 3).

Cells Overexpressing Interleukin 10 Receptor or Its Presumptive Downstream Target Mcl1 Are Selected during B-Lymphomagenesis

The approach described above was applied to determine whether levels of IL10R are rate-limiting during B-lymphomagenesis. IL10R α full-length cDNA is described in MATERIALS AND METHODS. As a negative control, we also generated and utilized its truncated version (IL10R_{STOP}) retaining the extracellular and transmembrane domains but lacking the cytoplasmic tail (see MATERIALS AND METHODS). Transfection of both constructs into the packaging cells resulted in expression of polypeptides of expected lengths (immunoblotting data not shown) located on the cell surface (flow cytometry data in Fig. 4A). The IL10R and IL10R_{STOP} retroviruses were used to infect Myc3 cultures as described in the previous section.

When transduced cells were used for tumor production *in vivo*, a steady increase in the percentages of IL10R-overexpressing, GFP-positive cells was apparent: after only three passages *in vivo* such cells accounted for over 90% of total neoplastic cells, up from 10% observed after infection *in vitro* (Fig. 4B). No increase was observed with GFP- or IL10R_{STOP}-overproducing cells. Additionally, no increase was observed when the experiment was performed in IL10-deficient mice,²⁹ suggesting that the positive selection for IL10R-overexpressing cells depends on the availability of its cognate ligand.

IL-10 is known to signal predominantly through the STAT3 pathway (reviewed in Ref. 30), which activates expression of several anti-apoptotic genes including Bcl-2, Bcl-xl, survivin, and Mcl1.³¹ Consistent with this, Mcl1 was upregulated in Myc^{OFF} tumors, albeit slightly less than fourfold (data not included in Table 2). We thus asked whether positive selection for Mcl1-overexpressing cells would also be in place. We generated a murine Mcl1/GFP-encoding retrovirus and repeated the basic experiment. As evidenced by data in Figure 4C, after only

two passages *in vivo*, Mcl1-transduced cells accounted for almost 100% of the total cell population. Thus, it is possible that the contribution of the IL10 pathway to B-lymphomagenesis is based on its ability to inhibit apoptosis and improve tumor-cell survival. Consequently, targeting of the IL10 pathway using soluble IL10R α ³² or a dominant-negative STAT3³³ could block expansion of lymphoma cells. Finally, functional validation of other Myc-regulated genes, which is under way in our laboratory, might yield new therapeutic targets.

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FIGURE 1.

The non-transgenic mouse model of B-cell lymphoma based on the expression of conditionally active Myc in *p53*-null bone marrow cells. The use of *4-OHT* deprivation allows the analysis of the role of Myc in tumor maintenance.

FIGURE 2.

MycER activity correlates with rapid tumor growth and high rates of apoptosis. Plots refer to tumor sizes in animals continuously treated with the estrogen (with 4OHT), deprived of the estrogen (w/o 4OHT), and retreated with the estrogen after a 2-week hiatus (w/o > with 4OHT). The insets depict TUNEL staining performed on representative tumor samples from each group.

FIGURE 3.

An *in vivo* selection assay for cells overexpressing Myc target genes. See text for more explanations.

FIGURE 4.

(A) Retrovirus-driven expression of IL10R and IL10R_{stop} on the surface of transfected BOSC23 cells. Live cells were stained with an anti-IL10R antibody as described in MATERIALS AND METHODS and analyzed using flow cytometry (APC fluorophor). (B) Positive selection for cells overexpressing IL10R. Percentages of GFP-positive cells in Myc3 B-cell lymphoma cells transduced with either empty vector (GFP) or IL10R/GFP and IL10R_{stop}/GFP-expressing retroviruses. 0' refers to cells passaged *in vitro*. 1', 2', and 3' refer to tumors propagated *in vivo* for the indicated number of passages. The last panel depicts the IL10R selection experiment performed in IL10-null hosts. (C) Positive selection for cells overexpressing Mcl1. All designations are the same as in B.

TABLE 1

Myc-activated genes

Fold Induction	Affy ID	Common name	Description
38.2	97473_at	Tn4sf7	transmembrane 4 superfamily member 7
24.4	102632_at	Calmbp1	calmodulin binding protein 1
14.3	96293_at	2410015N17Rik	RIKEN cDNA 2410015N17 gene
13.7	94971_at	Cdkn3	cyclin-dependent kinase inhibitor 3
12.9	104423_at	2810047L02Rik	RIKEN cDNA 2810047L02 gene
7.4	96269_at	Idi1	isopentenyl-diphosphate delta isomerase
6.5	97124_at	Finl5	fibroblast growth factor inducible 15
6.5	103525_at	Hmrpl1	heterogeneous nuclear ribonucleoprotein L-like ect2 oncogene
6.4	97411_at	Ect2	
6.1	103203_f_at	4432406C08Rik	RIKEN cDNA 4432406C08 gene
6.1	160159_at	Ccnb1	cyclin B1
5.6	160501_at	Kif20a	kinesin family member 20A
5.4	92840_at	Nup54	nucleoporin 54
5.3	95755_at	Csda	cold-shock domain protein A
5.3	160906_i_at	E430003D02Rik	RIKEN cDNA E430003D02 gene
5.3	99578_at	Top2a	topoisomerase (DNA) II alpha
5	93099_f_at	Plk	polo-like kinase homolog (Drosophila)
5	160395_at	D11Erd603e	DNA segment, Chr 11, ERATO Doi 603, expressed
5	93250_r_at	Hmgb2	high-mobility group box 2
5	101065_at	Pena	proliferating cell nuclear antigen
4.9	160069_at	Gmin	geminin
4.9	94292_at	Strap	serine/threonine kinase receptor-associated protein
4.7	97527_at	Cks2	CDC28 protein kinase regulatory subunit 2
4.7	101589_at	Hmg2	high-mobility group nucleosomal binding domain 2
4.7	100612_at	Rrm1	ribonucleotide reductase M1
4.6	99457_at	Mki67	antigen identified by monoclonal antibody Ki 67
4.5	92807_at	Txn1	thioredoxin 1
4.5	100128_at	Cdc2a	cell division cycle 2 homologue A (S. pombe)
4.5	94255_g_at	Clic4	chloride intracellular channel 4 (mitochondrial)
4.4	102911_at	Brc2	breast cancer 2
4.4	97468_at	Cks1	CDC28 protein kinase 1
4.3	100116_at	2810417H13Rik	RIKEN cDNA 2810417H13 gene
4.3	94274_at	Ube2s	ubiquitin-conjugating enzyme E2S
4.3	96784_at	Anln	anillin, actin-binding protein (scraps homolog, Drosophila)
4.3	94953_at	Racgap1	Rac GTPase-activating protein 1
4.2	100512_at	Uchl5	ubiquitin carboxyl-terminal esterase L5
4.2	160955_at	2010309E21Rik	RIKEN cDNA 2010309E21 gene
4.2	101521_at	Birc5	baculoviral IAP repeat-containing 5 (survivin)
4.2	92790_at	Kpna2	karyopherin (importin) alpha 2
4.2	104097_at	Bub1	budding uninhibited by benzimidazoles 1 homologue (S. cerevisiae)
4.1	94294_at	Ccnb2	cyclin B2
4.1	101959_r_at	Tfdp1	transcription factor Dp 1
4	92639_at	Stk6	serine/threonine kinase 6
4	99098_at	Fdps	farnesyl diphosphate synthetase

TABLE 2

Myc-repressed genes

Fold repression	Affy ID	Common name	Description
-21.8	99446_at	CD20 (a.k.a. Ms4a1)	membrane-spanning 4 domains, subfamily A, member 1
-21.7	96109_at	Klf2	Kruppel-like factor 2 (lung)
-21	103061_at	Gad1	glutamic acid decarboxylase 1
-20.5	96515_at	Il4i1	interleukin-4 induced 1
-20.4	98034_at	H2-DMb1	histocompatibility 2, class II, locus Mb1
-18.9	96525_at	Il10ra	interleukin-10 receptor, alpha
-17.1	96207_at	Rbms1	RNA binding motif, single stranded interacting protein 1
-16.7	160667_at	Evl	Ena-vasodilator stimulated phosphoprotein
-16.5	103985_at	Map4k2	mitogen-activated protein kinase kinase kinase 2
-16.1	101845_s_at	Cspgs	component of Sp100-rs
-13.6	102940_at	Ldb	lymphotoxin B
-13.4	103299_at	Al132321	expressed sequence A1132321
-10.8	102378_at	Sspn	Sarcospan
-10.2	94354_at	Abca1	ATP-binding cassette, sub-family A (ABC1), member 1
-9.7	103422_at	Cd1d1	CD1d1 antigen
-9	98859_at	Acp5; TRAP	acid phosphatase 5, tartrate resistant
-8.8	98974_at	Igh-4	similar to immunoglobulin delta-chain
-8.7	93714_f_at	LOC436489	similar to MHC class I-alpha
-8.4	100011_at	Klf3	Kruppel-like factor 3 (basic)
-7.8	92471_i_at	Sfn2	schlafen 2
-7.8	95373_at	Cd2	CD2 antigen
-7.7	101792_at	H2-Aa	Mouse-gene fragment for delta-immunoglobulin (exon 8)
-7.6	92866_at	Dgka	histocompatibility 2, class II antigen A, alpha
-7.3	103596_at	Macf1	diacylglycerol kinase, alpha
-7.3	98402_at	Gbp3	microtubule-actin crosslinking factor 1
-7.2	103202_at	Irf205	guanylate nucleotide binding protein 3
-6.8	94224_s_at	Irf205	interferon-activated gene 205
-6.6	92740_at	Iga	mouse Ig germline D-I-C region alpha gene and secreted tail.
-6.6	103697_at	A230075M04Rik	RIKEN cDNA A230075M04 gene
-6.1	99511_at	Prkcb	protein kinase C, beta
-6.1	160754_at	Pygm	muscle glycogen phosphorylase
-5.9	100998_at	H2-Ab1	histocompatibility 2, class II antigen A, beta 1
-5.7	101912_at	I100001G20Rik	RIKEN cDNA 1100001G20 gene
-5.5	101054_at	Ii	Ia-associated invariant chain
-5.5	93683_at	Rag1	recombination activating gene 1
-5.4	94834_at	Ctsh	cathepsin H
-5.3	98035_g_at	H2-DMb1	histocompatibility 2, class II, locus Mb1
-5.2	94713_at	Myo7a	myosin VIIa
-5.1	104429_at	H2-Ob	histocompatibility 2, O region beta locus
-5	93026_at	Mgst1	microsomal glutathione S-transferase 1
-5	103531_f_at	I300013B24Rik	RIKEN cDNA 1300013B24 gene
-5	93657_at	Spib	Spi-B transcription factor (Spi-1/PU.1 related)
-4.9	97313_at	Gdi1	guanosine diphosphate (GDP) dissociation inhibitor 1
-4.8	99623_s_at	Olfm1	olfactomedin 1
-4.8	93104_at	Btg1	B-cell translocation gene 1, anti-proliferative
-4.8	101048_at	Piprc	protein tyrosine phosphatase, receptor type, C
-4.8	93688_at	Cmah	cytidine monophospho-N-acetylneuraminic acid hydroxylase
-4.7	98001_at	Arhgef1	Rho guanine nucleotide exchange factor (GEF) 1
-4.5	92436_at	Slk23	serine/threonine kinase 23
-4.5	160781_r_at	Unc93b1	unc-93 homologue B1 (C. elegans)
-4.4	100295_at	Lgl	mouse Ig germline lambda-chain gene Vx-J2-C2-region, clone 30X2.
-4.4	161819_f_at	Laptn5	lysosomal-associated protein transmembrane 5
-4.4	99953_at	Rgl2	rat guanine nucleotide dissociation stimulator-like 2
-4.3	161005_at	5730420B22Rik	RIKEN cDNA 5730420B22 gene

Fold repression	Affy ID	Common name	Description
-4.3	94285_at	H2-Eb1	histocompatibility 2, class II antigen E beta
-4.2	93321_at	Ifi203	interferon activated gene 203
-4.2	95119_at	I110038D17Rik	RIKEN cDNA I110038D17 gene
-4.2	160812_at	Gga2	Golgi-associated, gamma adaptin ear containing, ARF-binding protein 2
-4.2	98980_at	Cd37	CD37 antigen
-4.1	97963_at	Sipa1	signal-induced proliferation associated gene 1
-4.1	160773_at	Zcche7	zinc finger, CCHC domain containing 7
-4.1	97173_f_at	H2-K2; H-2K2	similar to MHC class I-alpha
-4.1	95893_at	Blk	B lymphoid kinase
-4.1	98931_at	Gns	glucosamine (N-acetyl)-6-sulfatase
-4.1	101331_f_at	Igk-V8	immunoglobulin kappa chain variable 8 (V8)
-4.1	93092_at	H2-DMa	histocompatibility 2, class II, locus DMa
-4.1	100511_at	6330406L22Rik	RIKEN cDNA 6330406L22 gene
-4	103970_at	Dri2	dead ringer homologue 2 (Drosophila)