IDENTIFICATION OF GLUCOCORTICOID REGULATED MICRORNAS IN HUMAN ACUTE LYMPHOBLASTIC LEUKEMIA CELLS

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DOCTORAL THESIS

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Abstract

Background:
Glucocorticoids (GC) have pronounced effects on metabolism, differentiation, proliferation and cell survival in many tissues. In cells from the lymphoid lineage they induce massive apoptosis which led to their inclusion in essentially all chemotherapy protocols for lymphoid malignancies, particularly childhood acute lymphoblastic leukemia (ALL).
MicroRNAs (miRNAs) are $\sim$22 nucleotide RNA molecules that control essential biological functions including proliferation, differentiation and apoptosis. miRNAs act as post-transcriptional repressors of their target genes by either inhibiting their translation, or by directly cleaving and thus degrading their mRNA.
Essentially all effects of GCs are mediated via its receptor, the glucocorticoid receptor (GR), a transcription factor regulating a plethora of genes. Whether also miRNA genes are regulated by GCs is not known. Thus we investigated miRNA expression and regulation at the various stages of miRNA biogenesis in GC treated CCRF-CEM T-ALL cells and performed GO analysis on their target genes to reveal their potential functional role.

Results:
Three miRNAs, miR-15b, miR-16 and miR-223 were identified to be regulated at pri-, pre- and mature levels after GC treatment. Two other miRNAs, miR-19a and miR-92-1, were regulated at pri- and pre-miRNA levels, but no regulation was detected at mature level, implicating a high stability of the mature miRNA, or failed detection on the Ambion due to technical problems. Prediction of target genes resulted in 1208 genes potentially post-transcriptionally repressed by the GC regulated miRNAs. Target genes did not show significantly decreased mRNA levels indicating that most target genes are repressed by translational repression rather than by mRNA cleavage. GO analysis of the target genes led, in concordance with previous observations, to the conclusion that the GC regulated miRNAs are involved in cell cycle arrest, cell differentiation and apoptosis. Identification of *regulation of transcription* among the enriched biological processes could indicate a potential regulatory feedback loop of the GR mediated by the induced miRNAs.

Conclusion:
For the first time it has been shown that GCs induce expression of miRNAs. The comprehensive analysis of pri- pre- and mature miRNA expression revealed 3 miRNAs, miR-15b, miR-16 and miR-223 to be induced by GCs in ALL cells. GO analysis and previous reports suggest the involvement of the GC regulated miRNAs in GC apoptosis and cell cycle arrest. Whether their contribution is required for, or facilitates or accelerates, GC induced apoptosis has to be revealed by experimental investigations.

Keywords: glucocorticoids, acute lymphoblastic leukemia, microRNA, expression profiling

Publications

This thesis is based on the following publications:

Rainer J, Jesacher S, Ploner A, Niederegger H, Ploner C and Kofler R. Glucocorticoids regulate microRNAs-223 and 15b∼16-2 in human acute lymphoblastic leukemia cells. 2007. submitted.

Ploner C, **Rainer J**, Niederegger H, Eduardoff M, Villunger A, Geley S and Kofler R. The BCL2 rheostat in glucocorticoid-induced apoptosis of acute lymphoblastic leukemia. 2007. submitted.

Rainer J, Sanchez-Cabo F, Stocker G, Sturn A and Trajanoski Z. CARMAweb: comprehensive R- and bioconductor-based web service for microarray data analysis. *Nucleic Acids Res*, 2006. 34(Web Server Issue):W498-503.

Schmidt S, **Rainer J**, Riml S, Ploner C, Jesacher S, Achmuller C, Presul E, Skvortsov S, Crazzolara R, Fiegl M, Raivio T, Janne O A, Geley S, Meister B, Kofler R. Identification of glucocorticoid-response genes in children with acute lymphoblastic leukemia.*Blood*, 2006. 107(5):2061-2069.

Ploner C, Schmidt S, Presul E, Renner K, Schrocksnadel K, **Rainer J**, Riml S and Kofler R. Glucocorticoid-induced apoptosis and glucocorticoid resistance in acute lymphoblastic leukemia. *J Steroid Biochem Mol Biol*, 2005. 93(2-5):153-160.

Schmidt S, **Rainer J**, Ploner C, Presul E, Riml S and Kofler R. Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance. *Cell Death Differ*, 2004. 11 Suppl 1:S45-55.

Sturn A, Mlecnik B, Pieler R, **Rainer J**, Truskaller T, Trajanoski Z. Client-Server Environment for High-Performance Gene Expression Data Analysis. *Bioinformatics*, 2003. 19:772-773.

Contents

Background

Glucocorticoids (GC) have pronounced effects on metabolism, differentiation, proliferation and cell survival in many tissues. In malignant lymphoid cells these steroid hormones affect cell cycle progression and induce massive apoptosis. These anti-leukemic effects led to their inclusion in essentially all chemotherapy protocols for lymphoid malignancies, particularly childhood acute lymphoblastic leukemia [1]. Despite being used as therapy in childhood leukemia since the early 1960ies, the precise way GCs induce apoptosis in malignant ALL cells and the molecular basis of GC sensitivity and, the clinically relevant, *in vivo* GC resistance remain still elusive [2, 3, 4].

Most, if not all, effects of GCs are mediated via its cognate receptor (GC receptor (GR)), a ligand-activated transcription factor of the nuclear receptor super-family that resides in the cytoplasm and, upon ligand binding, translocates into the nucleus and starts its transcriptional program. The transcriptional response to GCs comprises induction and repression of many coding mRNAs, including pro- and anti-apoptotic genes, which have been identified in various model systems (reviewed in [2]) and related clinical samples [1, 5]. Some of these genes are currently functionally tested to further elucidate their role in GC induced apoptosis.

MicroRNAs (miRNAs) are small ∼22 nucleotide (nt) long non-coding RNA molecules that control essential biological functions including proliferation, differentiation and apoptosis. Disregulation of miRNAs has been associated with cancer [6, 7]. miRNAs are either embedded in introns of protein-coding or non-coding transcripts (intronic miRNAs) or constitute independent non-coding RNAs transcribed from their own promoters (exonic miRNAs) [8]. miRNAs are transcribed as mono- or polycistronic primary precursor transcripts (pri-miRNA), that are structurally analogous to mRNA (including intron-exon structure) [9]. Transcription of miRNA genes is mediated by polymerase II [10, 8]. The stem-loop structure within the pri-miRNA is cleaved by a complex consisting of the RNase III Drosha and the DiGeorge syndrome critical region 8 (DGCR8) proteins to release the ∼60nt hairpin, termed pre-miRNA [9]. The pre-miRNA is exported to the cytoplasm by the Ran-dependent nuclear transport receptor exportin-5 (Exp5). In the cytoplasm the pre-miRNA is further processed by a complex consisting of the cytoplasmic RNase III Dicer and HIV-1 TAR RNA-binding protein (TRBP) into a ∼22nt double stranded RNA (dsRNA). The mature ∼22nt long miRNAs are incorporated into the effector complexes, which are known as *miRNP*, *mirgonaute* or, more generally *miRISC* (miRNA-containing RNA inducing silencing complex) [8]. During miRISC assembly, the ∼22nt duplexes are rapidly converted into single strands (the mature miRNA). The miRISC can repress gene expression by two main posttranscriptional mechanisms: mRNA degradation (by direct mRNA cleavage or influencing the mRNA stability) or translational repression (inhibition of translation initiation, elongation or termination, as well as sequestering the mRNA in P-bodies, thus hiding them from the translation apparatus [11]). An almost complete complementary of the mature miRNA with the target mRNA is assumed to lead to mRNA cleavage [11]. After the cleavage of the mRNA, the miRNA remains intact and can guide the recognition and destruction of additional messages [12].

Based on their biogenesis, miRNAs can be detected at three different stages: 1) as long polyadenylated pri-miRNAs, 2) ∼60nt long pre-miRNAs and 3) ∼22nt long mature miRNAs (see figure 1). Besides the transcription of the pri-miRNA, extensive posttranscriptional regulations can influence the generation of the mature miRNA (e.g. RNA editing of the pri-miRNA by ADAR can inhibit the processing of the pri-miRNA by Drosha [13]). In principle miRNAs can be regulated at any step in their maturation process [14, 15]. Moreover RNA editing of the mature miRNA sequence, which has a major impact on the function of the miRNA, has been reported [16].

Since miRNAs post-transcriptionally regulate protein coding genes, identification of their target genes is crucial for the assessment of their possible function. So far only a few experimentally validated targets have been reported and computational analyses have to be performed in order to identify potential targets based on sequence similarities of the mature miRNA and their binding site in the 3' UTRs of the target genes [17, 18, 19, 20].

In lymphatic malignancies several miRNAs have been detected disregulated, like the miR-15a miR-16-1 cluster which is deleted in most patients with chronic lymphocytic leukemia or the miR-17∼92 cluster which is upregulated in 65% of B-cell lymphoma samples (reviewed in [6, 7, 21]). However, whether miRNAs are regulated by GC, either directly by the transcription fac-

tor GR or downstream to the GC response, has not been addressed so far, although miRNAs, by their virtue (i.e. repression of anti-apoptotic BCL2 by miRNAs miR-15 and miR-16 [22]), could contribute to the anti-leukemic effects of GCs.

We initiated therefore a search for GC regulated miRNAs in CCRF-CEM cells, a well studied *in vitro* model system for GC-induced leukemia apoptosis. Since regulation of miRNAs can occur at the different stages of the miRNA biogenesis, data from all three maturation steps were considered in the analysis. To reveal possible functional consequences of regulated miRNAs, mRNA target genes were identified, and, to determine whether the target genes are repressed by mRNA cleavage or mRNA destabilization (both reflected by altered mRNA levels), changes in mRNA expression of the target genes were evaluated.

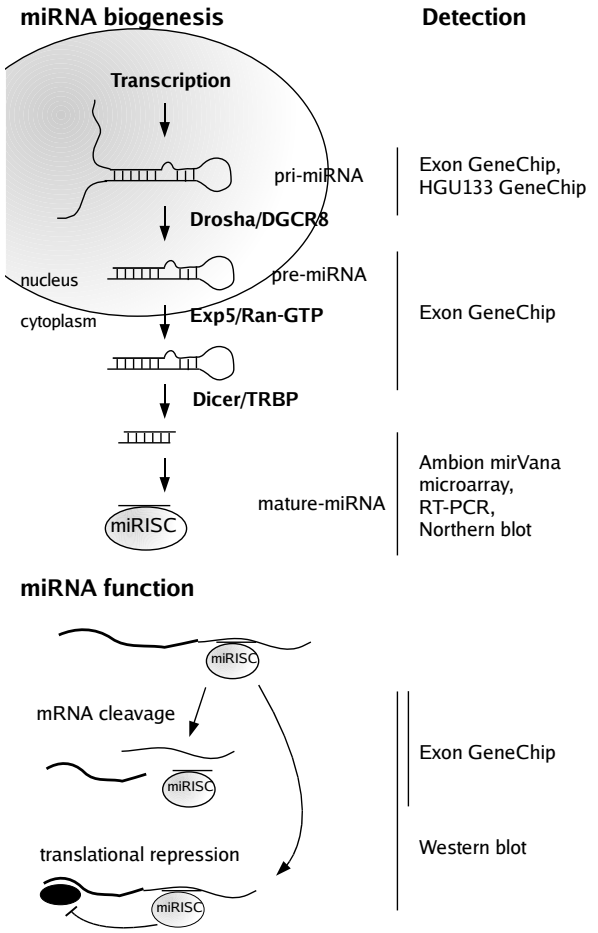


Figure 1: miRNA biogenesis and function (description in the main text). Detection: systems employed to detect the various intermediate (pri- / pre-miRNA) and end products (mature miRNA), as well as miRNA target mRNA (Exon GeneChip) and protein levels (Western blot).

Results

Glucocorticoids regulate miRNAs

To investigate whether GC regulate miRNAs, the GC-sensitive T-ALL cell line CCRF-CEM-C7H2 [23], was treated in three independent experiments with 100nM dexametasone (a GC) or EtOH as empty vehicle control and samples were taken at different timepoints (at 2, 6 and 24 hours for the Ambion mirVana^{TM}system for the detection of mature miRNAs and at 0.5, 2, 6 and 24 hours for the detection of mRNAs, pre- and pri-miRNAs using the Affymetrix Exon system).

Regulated mature miRNAs

For the detection of mature miRNA sequences the mirVana^{TM}miRNA Bioarrays version 1 system has been exploited. For a detailed description of the analysis see [24]. In brief, pooled RNA from 3 biological replicates per timepoint and treatment (2, 6 and 24 hours treatment with either GC or EtOH) were hybridized employing a dye-swap design onto, in total, 6 microarrays (each dye-swap-pair onto the same glass surface in two seperatly

hybridizable subgrids). miRNAs whichs features exhibited a more then 2-fold up- or downregulation (log2 ratio comparing GC treated against EtOH treated sample $M > 1$ or < -1) after dye-swap normalization in both replicated spots per array were called *regulated* (see table 1 for the list of regulated miRNAs).

To reconfirm the regulations seen on the microarrays, quantitative real time RT-PCRs and northern blot analyses of all regulated miRNAs were performed, but only the *late* regulations (24 hours GC treatment) could be verified [24] (although two independent RT-PCR systems were used for the early timepoints). Hence we concluded that GC induce the expression of the 3 mature miRNAs miR-15b, miR-16 and miR-223.

miRNA	M_{2h}	M_{6h}	M_{24h}	A_{2h}	A_{6h}	A_{24h}
miR-15b	0.57	0.70	**1.37***	10.1	10.0	10.8
miR-16	0.69	0.88	**1.18***	13.2	12.8	13.8
miR-181a	0.88	**1.11**	0.73	12.9	12.7	13.2
miR-19b	**1.36**	**1.42**	0.26	11.2	10.7	11.8
miR-223	0.45	0.22	**1.25***	9.8	9.5	10.5

Table 1: GC-regulated mature miRNAs. M_{2h}, M_{6h}, M_{24h}, A_{2h}, A_{6h} and A_{24h}: log2 fold change values (M) and average expression values (A) after 2, 6 and 24 hours treatment respectively (average values of both replicated spots per array). Regulated miRNAs are highlighted. *: verified with RT-PCR and/or northern blot.

Regulated pre-miRNAs

Since miRNAs can be regulated at each stage of their biogenesis [14, 15], we further analyzed expression profiles of the CCRF-CEM-C7H2 cell line performed on the Affymetrix Exon chip (HuEx 1.0 ST), to possibly identify additional regulated pre-miRNAs, not detected by the previous analysis. Missing detection could be caused 1) by technical problems during sample preparation, hybridization or data processing, 2) by missing probes on the Ambion arrays or 3) by lacking Dicer processing of the pre-miRNA. Mature miRNAs can not be detected with the Exon system since they are lost during sample preparation due to their small size, but pri-miRNAs and pre-miRNAs could be detected because the RNA used for target labeling is randomly primed and, according to our analysis, the Exon chip contains several oligonucleotide probes targeting pre-miRNA sequences (see Material and Methods).

To identify GC regulated pre-miRNAs a dataset was analyzed consisting of 24 Exon chips with samples from the cell line CCRF-CEM-C7H2 treated for 0.5, 2, 6 and 24 hours either with GC or, as control, with EtOH. Each treatment/timepoint is represented by 3 biological replicates derived from 3 independent experiments. From the 259 pre-miRNAs detectable on Exon chips only one pre-miRNA is significantly regulated (false discovery rate (FDR) ¡ 5%) at the early treatment timepoints (see figure 2 and table 2). The miR-637 precursor is already regulated after 0.5 hours treatment ($p_{BH} = 0.027$, $M = 1.08$; regulation disappears at later treatment timepoints). Since this pre-miRNA is represented by just one 25nt oligo probe with a high guanine-cytosine content (15/25), thus making the probe susceptible to cross-hybridization, and due to the missing regulation of its pri-miRNA this regulation was not further considered. The pre-miR-675, regulated significantly after 6 and 24 hours GC treatment, is also represented by a single probe only. This regulation was also not further evaluated, since its published pri-miRNA (H19 [25]) shows no altered expression due to GC treatment (see figure 3(b)). To proof that these regulations are indeed artificial (e.g. due to cross-hybridization of the oligo probes) RT-PCR verification would have to be performed.

Pre-miRNAs of miR-181a and miR-19b (miR-181a-1, miR-181a-2 and miR-19b-1, miR-19b-2 respectively) were not differentially expressed in any of the 4 treatment timepoints (data not shown), providing further evidence, besides lacking verification with RT-PCR and northern blot, that their regulation as detected on the Ambion arrays was artificial. Taken together 4 miRNAs, miR-15b, miR-223, miR-19a and miR-92-1 were identified to be regulated by GC at the pre-miRNA level, and two of them, miR-15b and miR-223, also at the mature level. Pre-miR-16-2 (part of the miR-15b miR-16-2 cluster, and regulated at the mature level) was not significantly regulated, but still was the next best regulated miRNA after those listed in table 2 with an $p_{BH} = 0.34$ and $M = 0.47$ after 24 hours treatment.

Regulated pri-miRNAs

According to the current understanding miRNA genes are transcribed as long polyadenylated primary transcripts (pri-miRNAs) from their genomic locus. To identify differentially expressed pri-miRNAs and to validate if the regulation of the previously defined GC reg-

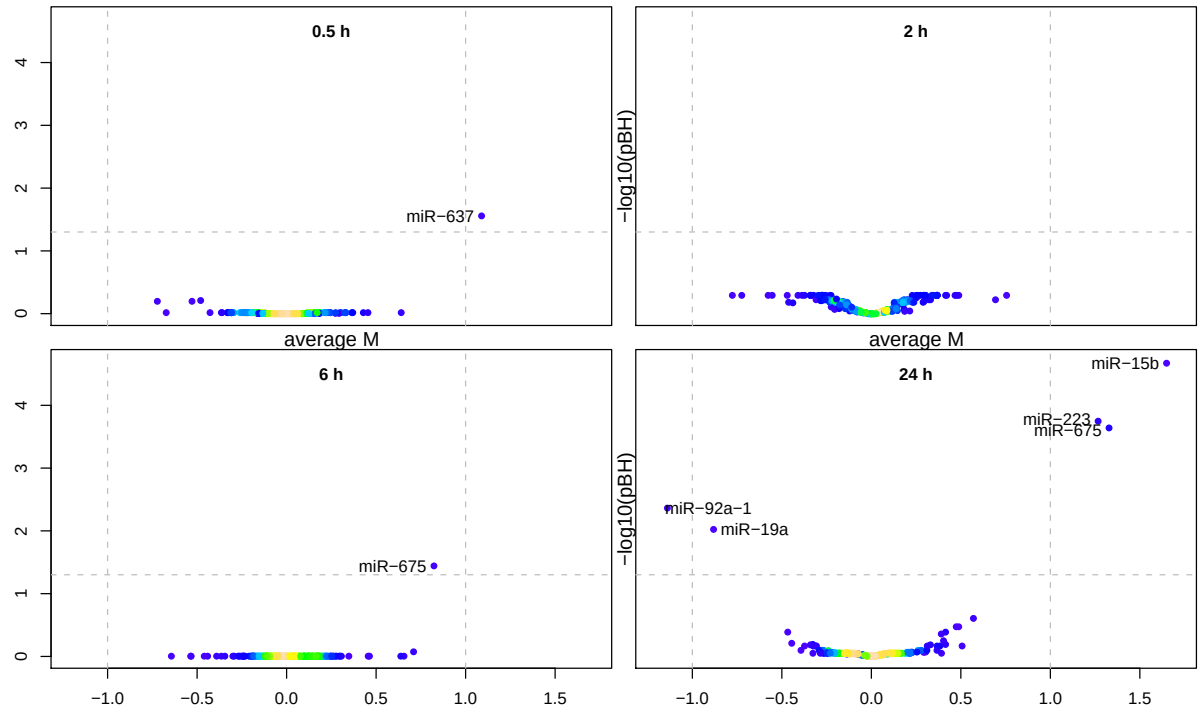


Figure 2: Volcano plots (average log2 fold change (M) on the X- against significance of differential expression ($-log_{10}$ of the Benjamini Hochberg adjusted p-value p_{BH}) on the Y-axis) of the pre-miRNAs detected on the Exon chips at different treatment timepoints of the CCRF-CEM-C7H2. Horizontal lines represent a $p_{BH} = 0.05$, differentially expressed pre-miRNAs ($p_{BH} < 0.05$) are represented by their name. Points are colored according to the local point density.

ulated miRNAs occurs at the transcriptional level, potential pri-miRNA transcripts were identified using the miRGen database [26] and Ensembls Biomart database (details in Material and Methods). Based on this analysis primary transcripts of 328 miRNAs have been identified. Potential pri-miRNAs of 201 of these miRNAs are detectable on the Affymetrix Exon array. These miRNAs are located within introns or exons of 169 pri-miRNAs (some pri-miRNAs constitute the primary transcript of two or more miRNA genes, since approximately 50% of known miRNAs are found in clusters and are transcribed as polycistronic primary transcripts [8, 27]).

Analysis of the same 24 Exon GeneChips, exploited in the identification of GC regulated pre-miRNAs, revealed that 32 pri-miRNA genes are differentially expressed after 24 hours GC treatment with a false discovery rate (FDR) smaller than 5%. At earlier treatment timepoints only pri-miR-223 was significantly regulated (after 2 hours, see table 3). Pri-miR-634 fell just below the cut-off for a significant regulation after 6 hours treatment ($p_{BH} = 0.06$). The extend of regulation of pri-miR-223 increased over time analogous to the regulation of its pre-miRNA (figure 3(a)). The quick response of this miRNA gene to GC treatment suggests direct transcriptional regulation of the pri-miRNA by the GR (currently under investigation by promoter studies). Correlations between pri- and pre-miRNAs of miR-223, miR-15b, miR-16-2, miR-92-1 and miR-19a are high (Pearson correlation, $R^2 = 0.977$, $R^2 = 0.908$, $R^2 = 0.994$, $R^2 = 0.935$ and $R^2 = 0.865$ respectively), implicating that the defined pri-miRNAs are indeed the primary transcripts of these miRNAs. Correlation of most other pri-/pre-miRNA pairs are poor or even negative implicating that a) prediction of pri-miRNAs was not appropriate for these miRNAs or b) affected pri-miRNAs are post-transcriptionally regulated (i.e. lack-

miRNA	$p_{BH0.5}$	$M_{0.5}$	$A_{0.5}$	p_{BH2}	M_2	A_2	p_{BH6}	M_6	A_6	p_{BH24}	M_{24}	A_{24}
miR-15b	1.00	0.02	5.6	0.73	0.18	5.8	0.84	0.71	6.2	**0.00**	**1.65**	**6.7**
miR-675†	0.96	0.33	6.5	0.51	0.48	6.7	**0.04**	**0.82**	**6.7**	**0.00**	**1.33**	**7.2**
miR-223	1.00	0.04	6.4	0.51	0.49	6.8	0.99	0.66	7.1	**0.00**	**1.27**	**7.0**
miR-19a	0.96	0.17	6.2	0.51	0.47	6.1	0.99	-0.64	6.8	**0.01**	**-0.88**	**5.9**
miR-92-1	1.00	0.05	7.2	0.60	0.32	7.2	0.99	-0.05	7.5	**0.00**	**-1.14**	**7.3**
miR-637†	**0.03**	**1.09**	**6.9**	1.00	0.03	6.9	0.99	0.05	6.9	0.62	-0.44	6.9

Table 2: GC regulated pre-miRNAs (ordered according to the extend of regulation) as detected on the Exon arrays. Significant regulations at the corresponding treatment timepoints are high-lighted. †: pre-miRNAs detected by only one 25nt oligo probe on the Exon array, all other pre-miRNAs are measured by 4 distinct oligo probes.

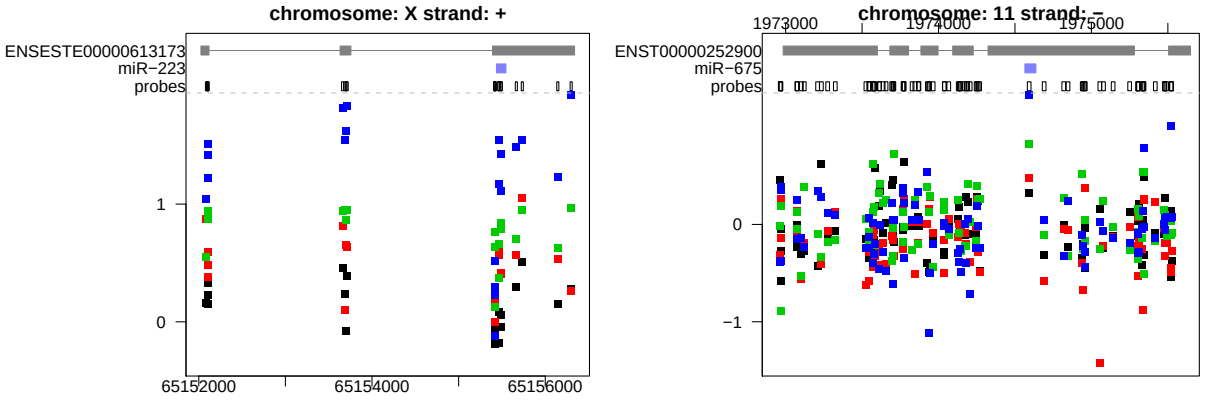
miRNA	symbol	$p^*_{0.5}$	$M_{0.5}$	$A_{0.5}$	p^*_2	M_2	A_2	p^*_6	M_6	A_6	p^*_{24}	M_{24}	A_{24}
miR-548d-1	ATAD2	0.86	0.09	8.0	0.38	0.13	8.2	0.47	0.25	8.5	**0.00**	**1.62**	**9.2**
miR-15b-16†	SMC4	0.99	-0.02	9.2	0.37	0.13	9.7	0.90	0.15	9.9	**0.00**	**1.46**	**10.6**
miR-223		0.86	0.17	7.4	**0.01**	**0.45**	**7.7**	0.26	0.77	7.6	**0.00**	**1.28**	**7.7**
miR-634	PRKCA	0.86	0.11	7.2	0.27	0.21	7.4	0.06	0.46	7.5	**0.00**	**0.97**	**7.6**
miR-621	SLC25A15	0.87	0.04	7.8	0.41	0.17	7.8	0.90	-0.12	7.9	**0.00**	**-0.96**	**7.6**
miR-17-92‡	C13orf25	0.86	0.08	7.1	0.27	0.20	7.0	0.90	-0.25	7.3	**0.00**	**-0.79**	**6.8**
miR-652	TMEM164	1.00	-0.01	7.7	0.41	0.15	7.9	0.90	0.10	7.7	**0.00**	**0.70**	**8.1**
miR-26a-2	CTDSP2	0.90	0.05	7.9	0.27	0.18	8.0	0.59	0.21	7.8	**0.00**	**0.70**	**8.3**
miR-128b	ARPP-21	0.86	0.14	8.7	0.36	0.11	8.8	0.90	0.08	8.8	**0.01**	**0.64**	**9.4**
miR-580	LMBRD2	1.00	-0.02	6.4	0.55	0.10	6.6	0.99	0.02	6.8	**0.01**	**0.63**	**7.2**
miR-32	C9orf5	0.99	0.02	8.8	0.37	0.19	9.1	0.26	0.28	9.1	**0.02**	**0.52**	**9.2**
miR-628	CCPG1	0.86	0.10	5.8	0.27	0.18	5.9	0.54	0.18	6.4	**0.02**	**0.52**	**6.4**

Table 3: GC regulated pri-miRNAs. 31 pri-miRNAs are significantly regulated at a false discovery rate smaller 5% after 24 hours GC treatment. The 12 miRNAs with the strongest regulations ($|M| > 0.5$) are presented here. p^*: p-values adjusted for multiple hypothesis testing using Benjamini and Hochbergs method for a strong control of the FDR. Values in bold font represent significant regulations at the corresponding timepoint. miR-15b-16†: pri-miRNA of miR-15b, miR-16-2 cluster. miR-17-92‡: pri-miRNA of miR-17, miR-18a, miR-19a, miR-19b-1, miR-20a, miR-92-1 cluster. Primary transcripts of miRNAs regulated at the pre-miRNA level are underlayed grey.

ing Drosha or Dicer processing).

For most of the differentially expressed predicted pri-miRNAs (table 3, pri-miR-548d-1, pri-miR-634, pri-miR-652, pri-miR-580 and pri-miR-628) neither pre- nor mature miRNA are detectable on Exon or Ambion system respectively. A regulation only identified at the pri-miRNA level is not evidence enough for a GC regulated miRNA, due to possible post-transcriptional regulation or inadequate assignment miRNA:pri-miRNA. Additional validation would have to be performed to evaluate a possible regulation of their mature miRNAs. Three other regulated potential pri-miRNAs (pri-miR-621, pri-miR-128b and pri-miR-32) are detectable at the pre-miRNA level, and, pri-miR-32, also on the mature level, but none of these miRNAs are regulated at any other level than that of the primary transcript. In this analysis we therefore focus on miRNA genes detectable and regulated at the precursor and mature levels.

Taken together the results from the analyses of differentially expressed mature, pre- and pri-miRNAs revealed that miR-223 and the miRNA cluster miR-15b, miR-16-2 are regulated by glucocorticoids in CCRF-CEM-C7H2 acute lymphoblastic leukemia cells and suggest that this regulation occurs at transcriptional level. Interestingly the pre-miRNA of miR-16-2 was not detected to be significantly differentially expressed ($p_{BH} = 0.34$, $M = 0.47$ after 24 hours treatment), although both its primary transcript (SMC4) and the mature form are regulated (regulation of the mature form also verified by RT-PCR and northern blot). Technical problems on the Exon array, either due to bad oligo probes or sample preparation and processing, most likely caused the lack of detection of the pre-miRNA regulation.



(a) pri-/pre-miR-223 (b) pri-/pre-miR-675

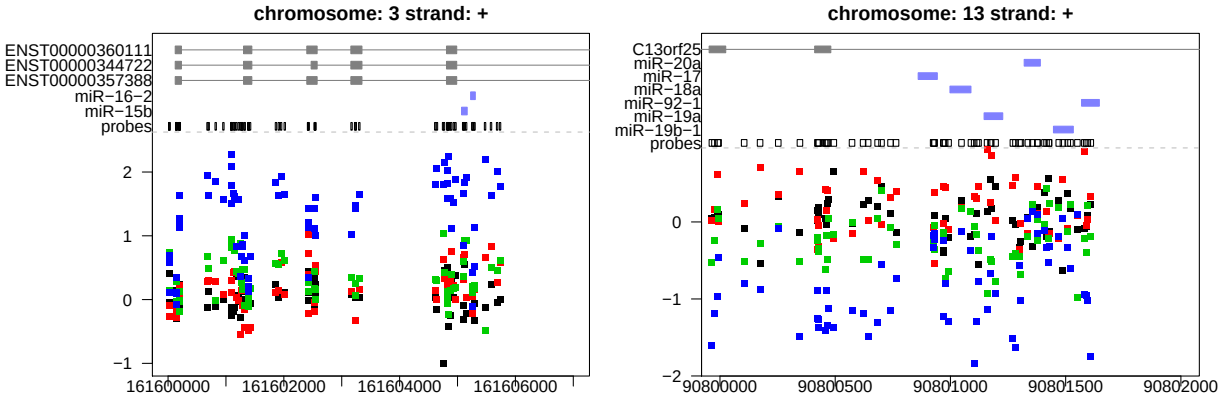
Figure 3: Genomic representation of pri/pre-miR-223 and pri/pre-miR-675 combined with their regulation (M values, y-axis) measured by the various exon probes. First row in both plots represents the pri-miRNA (grey), second row the location of the pre-miRNA (light blue) and third row the probes on the Exon array. In the lower part of the plot the M values (log2 fold change) of the probes at the various time points are presented (with black, red, green, and blue rectangles representing 0.5, 2, 6 and 24 hours treatment respectively). Both pri- and pre-miR-223 show clear and increasing regulation over time, whereas for miR-675 only a single probe mapping to the pre-miRNA is (increasingly over time) up-regulated, whereas all other probes surrounding the pre-miRNA-probe scatter around $M = 0$ (some probes also exhibiting downregulation).

The miRNAs miR-19a and miR-92-1, both part of the c-Myc regulated miR-17-92 polycistron with oncogenic potential [28], are significantly downregulated after 6 and 24 hours GC treatment at the pri- and pre-miRNA level, but not at the mature miRNA level. Thus could be caused either by technical problems in the detection of these miRNAs with the Ambion system, or due to high stability or long half life of the mature miRNA form. Other miRNAs from the miR-17-92 cluster are not significantly regulated (see also figure 4(b)). Besides technical reasons like oligo probe effect or losing the RNA during sample preparation or amplification, also a selective processing of the primary transcript, manifested e.g. by a lacking release of the (non-regulated) pre-miRNAs by Drosha after RNA editing of their respective stem loops in the primary transcript by ADARs[13], could be put forward.

Predicted targets of GC regulated miRNAs

In order to reveal the possible function of the previously identified GC regulated miRNAs (namely miR-15b, miR-16 and miR-223) and their potential contribution to GC induced apoptosis, their target mRNAs have to be predicted. Only a handful of experimentally verified miRNA targets have been discovered so far, therefore miRNA targets have to be identified by computational algorithms. Among the experimentally verified target genes are BCL2 for miR-15b and miR-16 [22] and NFIA for miR-223 [29] (for a comparison of target mRNA and miRNA regulation values see figure 5).

Most miRNAs repress their target genes by binding to specific binding sites in the 3' UTR of the genes mRNA. These miRNA target sites can be classified, based on experimental support, into three categories: 1) 5'-dominant canonical, 2) 5'-dominant seed only and 3) 3' compensatory [20, 30]. The first $\sim$7 nucleotides starting from the first or second nucleotide at the 5' end of the miRNA is defined as the seed region. Canonical sites have perfect base pairing in the seed region and extensive base pairing at the 3' end of the miRNA. The seed only sites have perfect base pairing at the seed region and limited base pairing at the 3' end of the miRNA. 3'-compensatory sites have extensive base pairing at the 3' end of the miRNA compensating for an imperfect base pairing in the seed region. miRNA target prediction algorithms make use of these miRNA-mRNA-binding models for the prediction of possible targets.



(a) miR-15b, miR-16-2 cluster

(b) miR-17-92 cluster

Figure 4: Genomic representation of the precursors of miRNA clusters miR-15b, miR-16-2 and miR-17-92 combined with their regulation (M values, y-axis) measured by the various exon probes. Left: upregulated pre-miR-15b and pre-miR-16-2 with part of their pimary transcript (SMC4). Right: downregulated pre-miR-19a and pre-miR-92-1 with part of their primary transcript C13orf25 and all miRNAs of the miRNA cluster (miR-17-92 cluster). In the lower part of both plots M values (log2 fold change) of the probes at the various time points are presented (with black, red, green, and blue rectangles representing 0.5, 2, 6 and 24 hours treatment respectively).

All popular target prediction programs (miRanda [17], TargetScanS [19] and PicTar [18]) require additional conservation of the target site across at least 2 species in order to reduce the very large portion of false positive predictions.

To generate a list of possible target genes for the GC regulated miRNAs a combination of available prediction algorithms was used, as proposed by Sethupathy et al. [20]. PicTar and TargetScanS both focus on 5'-dominant sites, whereas miRanda allows additionally the detection of 3' compensatory sites. Intersection of the predictions of the former two and union with the predictions of the latter algorithm results in the most complete list of miRNA-targets.

Combination of the 3 prediction algorithms yielded 1208 target genes of the GC regulated miRNAs (862 targets of miR-15b, 819 of mir-16 and 294 of miR-223, of which 830, 787 and 277 detectable on the Exon array). 52 genes are predicted targets of all 3 miRNAs (see figure 6). The high number of common target-genes for miRNAs miR-15b and miR-16 (702) is not surprising, since both miRNAs share a high sequence similarity and are transcribed from the same genomic locus, thus potentially have also the same function.

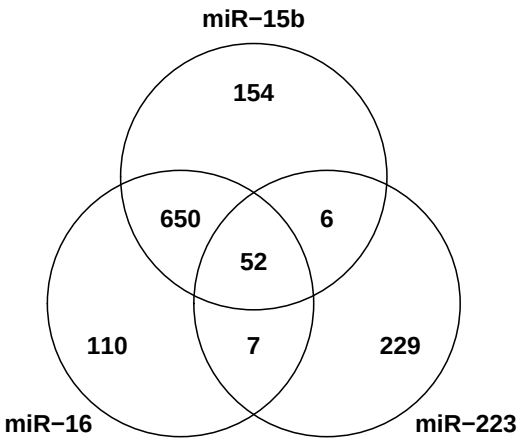
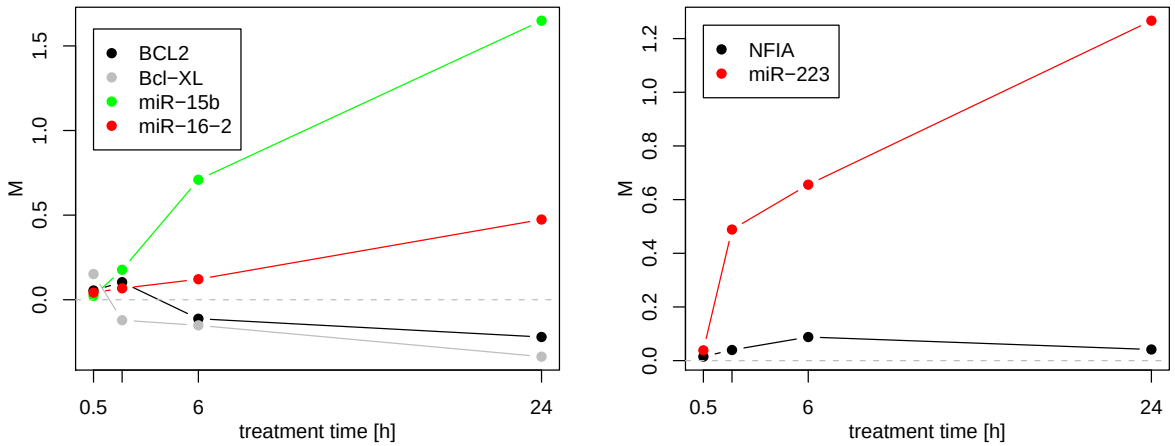


Figure 6: Venn diagram representing the number of predicted targets for each of the GC regulated miRNAs. 52 genes are predicted targets of all 3 miRNAs, whereas miR-15b and miR-16, sharing a high sequence similarity and being transcribed from the same genomic locus, have 702 target genes in common.



(a) miR-15b, miR-16, BCL2

(b) miR-223, NFIA

Figure 5: Regulation pattern of miR-15b and miR-16 (left) and miR-223 (right) and their experimentally verified targets. Left: verified target BCL2 and predicted target Bcl-XL mRNA levels seem to be slightly reduced by the induced miRNAs miR-15b and miR-16. Right: mRNA levels of verified miR-223 target NFIA show no apparent alterations, implicating that the gene might be regulated by translational repression rather than by mRNA cleavage.

Are the miRNA targets regulated at the mRNA level?

Besides translational repression (i.e. by influencing translation initiation, elongation or termination) miRNAs can repress target gene expression by direct mRNA cleavage or mRNA destabilization [11]. The latter post-transcriptional target regulation leads to decreased mRNA levels, which can be detected using microarrays [31, 32]. Thus we correlated the (increasing) regulation pattern of the GC regulated miRNAs with those of their predicted target genes. For the detection of mRNA expression the same dataset consisting of 24 Exon arrays of the cell line CCRF-CEM-C7H2 already exploited previously, could be analyzed, since Exon arrays allow a simultaneous detection of pre-miRNAs and mRNAs (including pri-miRNAs) in the same sample.

Predicted target genes of all 3 regulated miRNAs show a common temporal regulation pattern (see figure 7): on average all target genes of miR-15b, miR-16 or miR-223 exhibit a slightly increasing upregulation in the first two hours of treatment followed by a decrease in regulation and finally showing a minimal downregulation after 24h treatment. This slight downregulation of target genes detectable at mRNA level is in concordance to previous reports [31]. To reveal if this slight downregulation of the target genes at the mRNA level is significant ($M_{2h} - M_{24h} = -0.034$, p-value=0.00023) or if any set of genes exhibits the same downregulation, the difference of the M values after 2 and 24 hours GC treatment of the target genes (observed t-statistic) are compared to those of 10000 randomly selected gene sets (expected t-statistics). The p-value represents the fraction of expected t-statistics that are smaller then the observed. The null hypothesis, that the observed downregulation is not more pronounced than expected by chance, would be rejected at a p-value of 0.05. According to this analysis the slight downregulation of target genes as shown in figure 7 is not more significantly different than expected by chance (p-value 0.8397). Since multiple binding sites in the 3' UTR of target genes, either of the same miRNA or of co-regulated miRNAs, can boost target repression [18, 33] and hence could promote mRNA degradation, the analysis was repeated for the subset of 52 genes predicted to be targets of each GC regulated miRNA. But also this repression is not stronger than expected by chance ($p = 0.5125$).

Thus the GC regulated miRNAs seem to repress their target genes preferably by translational repression rather than by mRNA cleavage although mRNA cleavage could not be excluded for single target genes.

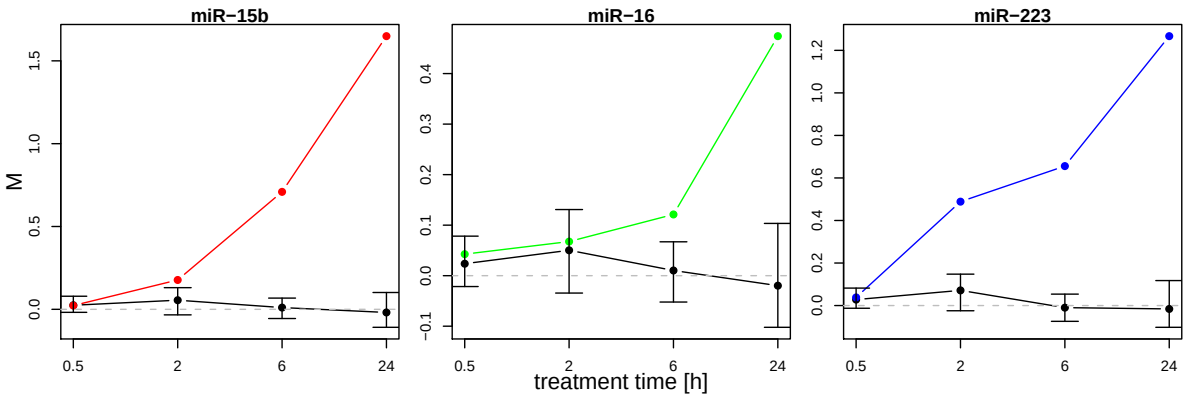


Figure 7: Regulation pattern of GC regulated pre-miRNAs and their target genes. Red, green and blue lines represent the M values at the 4 treatment timepoints of miR-15b, miR-16 and miR-223 respectively. For the target genes the median, 25% and 75% quartile of the M values are drawn. The targets of all 3 miRNAs show a characteristic (non-linear) pattern of a slight increasing upregulation in the first two treatment hours, followed by a continuous downregulation.

miRNA - transcription factor regulatory networks

Shalgi et al. proposed several models of combinatorial regulatory interactions of transcription factors (TF) and miRNAs [34]. To identify pairs of TFs and miRNAs that cooperate in regulating common target genes, they looked for TF-miRNA pairs with a high rate of cooccurence in the promoters and 3' UTRs of the genes. To reveal the existance of such potential regulatory networks in GC induced apoptosis, we identified target genes of miR-15b, miR-16 and miR-223 regulated after GC treatment. Such differential expression of a gene after GC treatment is, in contrast to the prediction of GR target genes based solely on sequence informations, an essential experimental support for the transcriptional regulation of the gene by the GR (either directly or by some TF downstream to GR). 213 of the 1090 predicted target gene transcripts detectable on the Exon array are regulated after 24 hours GC treatment at a FDR smaller 5%. Thus the GR and its regulated miRNAs potentially regulate common targets and constitute regulatory networks in the form of direct or indirect feed forward loops (FFL) as defined by [34]. Examples of these FFLs are the pro-apoptotic gene BCL2L11, upregulated by GR ($M_{24h} = 1.51$, $p_{BH24h} = 0.00004$) and a potential target of miR-16, TSC22D3 ($M_{24h} = 4.08$, $p_{BH24h} = 0.0000005$) and miRNAs miR-15b and miR-16, DDIT4 ($M_{24h} = 1.854$, $p_{BH24h} = 0.000014$) and miR-223 and SESN1 ($M_{24h} = 1.875$, $p_{BH24h} = 0.000013$) and miR-15b and miR-16. In all these FFL (*type I* or FFL TF $\rightarrow$ miRNA networks according to the definition of [34]) the TF (in this case the GR) induces expression of the miRNA and both act as regulators on the same target gene (the TF upregulating and the miRNA post-transcriptionally repressing the gene). With the exception of DDIT4 all target genes in the example FFLs are direct targets of GR (unpublished observation) and are among the most significantly regulated genes after GC treatment. Although such FFLs were identified for many TF-miRNA pairs, their functional implication is still elusive [34].

Potential functional role of the GC regulated miRNAs

To reveal the possible functional role of the GC regulated miRNAs a Gene Ontology (GO) [35] analysis was performed on the target genes of the GC regulated miRNAs. To identify the biological processes in which the target genes are involved, the number of target genes associated with any biological process GO term was compared to the number of genes from a background gene set also associated with the specific term. The background gene set was defined as the list of all genes predicted to be target of any currently known miRNA as identified by PicTar, TargetScanS or miRanda. The 1208 target genes of the GC regulated miR-

GO ID	p	count	size	term	15b†	16†	223†
GO:0051726	0.000	48	206	regulation of cell cycle	38	41	11
GO:0006350	0.000	117	632	transcription	85	86	42
GO:0016070	0.000	124	692	RNA metabolic process	90	86	40
GO:0007049	0.000	59	289	cell cycle	50	54	13
GO:0006355	0.001	76	406	regulation of transcription, DNA-dependent	52	52	25
GO:0043086	0.002	12	37	negative regulation of enzyme activity	10	10	2
GO:0006469	0.003	8	20	negative regulation of protein kinase activity	8	8	2
GO:0030163	0.004	16	59	protein catabolic process	12	13	5
GO:0022008	0.004	17	65	neurogenesis	15	13	4
GO:0030522	0.005	11	35	intracellular receptor-mediated signaling pathway	8	10	2
GO:0009790	0.006	15	56	embryonic development	12	11	5
GO:0030155	0.006	8	22	regulation of cell adhesion	6	5	2
GO:0045637	0.006	5	10	regulation of myeloid cell differentiation	3	3	4
GO:0007155	0.007	41	209	cell adhesion	37	31	15
GO:0000082	0.008	8	23	G1/S transition of mitotic cell cycle	7	8	0
GO:0045664	0.008	4	7	regulation of neuron differentiation	4	4	0
GO:0007369	0.008	4	7	gastrulation	2	1	1
GO:0043254	0.009	3	4	regulation of protein complex assembly	3	3	0
GO:0000079	0.009	7	19	regulation of cyclin-dependent protein kinase activity	6	7	3
GO:0030097	0.009	15	59	hemopoiesis	9	10	8

Table 4: Biological process GO terms enriched withtarget genes of any of the GC regulated miRNAs (p-value smaller 0.01). Count and size represent the number of target genes and number of genes from the background genes set associated with the GO term. †: number of target genes of the specific miRNA (miR-15b, miR-16 or miR-223) associated with the GO terms. Some target genes are predicted targets of two or all GC regulated miRNAs, thus sum of the numbers of the last 3 column is larger then the number stated in column *size*.

NAs are enriched in 60 biological process GO terms (p-value smaller 0.05; table 4 lists the 20 GO terms with $p < 0.01$).

Most of the enriched GO terms can be associated either with *transcription*, *cell cycle* or *cell development and differentiation*. The latter is in concordance with the current understanding of the miRNAs as regulators of cellular differentiation and required for the maintenance of the differentiated stage [36]. miR-223 has been described previously as a myeloid gene that is involved in hematopoietic lineage differentiation [37]. This is reflected by the GO terms *regulation of myeloid differentiation* (p-value for the over-representation of miRNA target genes at the specific GO term $p = 0.006$) and *hemopoiesis* ($p = 0.009$). Such myeloid specific signaling could have adverse effects in a lymphoid cell line (see discussion). Besides miR-223, which targets 4 genes involved in myeloid differentiation, also miR-15b and miR-16 are likely involved in myeloid differentiation both potentially regulating 3 target genes.

The most significantly enriched GO term is *regulation of cell cycle* ($p = 0.00006$, with 48 annotated predicted target genes). Particularly many target genes of miR-15b and miR-16 are annotated at this GO term, supporting previous observations that overexpression of the miR-16 cluster negatively regulates cellular growth and cell cycle progression [38].

A large fraction of predicted target genes of the GC regulated miRNAs are also involved in transcription and regulation of transcription (i.e. GO terms *transcription*, *RNA metabolic process* or *regulation of transcription, DNA dependent*). The GC regulated miRNAs thus could counteract the transcription initiated by the GR by repressing genes required for the transcription machinery. This would form a negative regulatory feedback loop in which the GR dampens its own transcriptional potential by activating factors inhibiting transcription.

Although not among the most significantly enriched GO terms also the GO terms *apoptotic mitochondrial changes* and *cell death* exhibit an over-representation of target genes. Most of the genes annotated to these processes are predicted targets of miR-15b and miR-16, which is in concordance with previous reports stating that the miR-15-16 cluster targets and represses the anti-

apoptotic gene BCL2 in vivo [39, 22]. Also our own results show slightly reduced mRNA levels of BCL2 after 24 hours (figure 5(a), slight but not significant downregulation $M_{24h} = -0.22$, $p_{BH24h} = 0.206$) and decreased BCL2 protein levels in the cell line CCRF-CEM-C7H2 after 36 hours GC treatment [40]. Also Bcl-XL, the anti-apoptotic splice variant of BCL2L1, and a predicted target of the GC regulated miRNAs, is downregulated at protein levels after 36 hours GC treatment, and exhibits slightly decreased mRNA levels after 24 hours treatment (5(a)). Other members of the BCL2 family among the predicted miRNA targets are the pro-apoptotic genes BCL2L11 (Bim), BAK1 and the anti-apoptotic genes MCL1 and BCL2L2 (Bcl-W). In contrast to BCL2 and Bcl-XL the pro-apoptotic BCL2 family genes show increased protein levels after 36 hours GC treatment (in line with the upregulation of BCL2L11 and on mRNA levels), implicating that the pro-apoptotic genes are either not repressed strongly enough or not at all by the miRNAs. Regulation of MCL1, which is slightly upregulated at mRNA level and also on protein levels [40], is not affected by the miRNAs. The regulatory potential of the miRNAs might be too weak to counteract the strong induction of these genes by the GR.

The GO analysis reflects recent, experimentally verified, findings of the functional roles of miR-15b, miR-16 and miR-223 and implicates a potential contribution of these miRNAs to characteristic processes promoted by GCs in ALL cells, namely cell cycle arrest and apoptosis [2, 41]. miR-15b and miR-16 have recently been described to be involved in apoptosis and cell cycle arrest [42, 38]. miR-223 has been implicated in hematopoietic differentiation with particular emphasis on the myeloid lineage [43, 37, 44]. This is in concordance with our finding of the enriched GO term *regulation of myeloid cell differentiation*. Induction of such myeloid specific differentiation signals might have adverse effects in the lymphoid lineage and could constitute a death signal. The regulation of genes involved in transcription could constitute a regulatory feedback loop that, depending on the balance of the process' repressors and activators regulated by the miRNAs, could facilitate or dampen transcription initiated by GR.

Knock down of these miRNAs in the presence of GC would reveal the *real* functional role of these miRNAs in combination with GCs and provide further evidence whether the miRNAs are required for GC induced apoptosis.

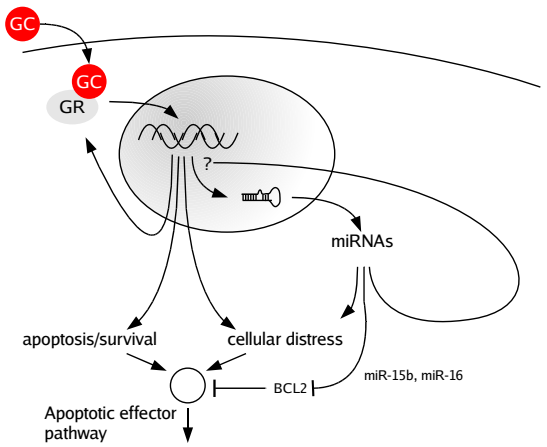


Figure 8: Proposed model for the contribution of GC regulated miRNAs to GC apoptosis. By promoting myeloid specific differentiation signals miR-223 could contribue to cellular distress. The miR-16 cluster has an experimentally verified impact on cell cycle arrest and apoptosis. By repressing BCL2 miR-15b and miR-16 could facilitate and accelerate GC induced apoptosis. Regulation of transcription of related genes could form a potential regulatory loop of GR mediated transcription. Whether this constitutes a positive or negative feedback loop depends on the balance between inhibitors and activators of transcription repressed by the miRNAs.

Conclusions and Discussion

The comprehensive analysis of the influence of GCs on miRNA expression, at both mature and precursor levels, demonstrated for the first time that GCs have the potential to regulate miRNAs. In the T-ALL cell line CCRF-CEM-C7H2 the mature forms of miRNAs miR-15b, miR-16 and miR-223 were identified to be induced after GC treatment. These miRNAs exhibit an increasing up-regulation with treatment duration, and the early response of miR-223 to GCs could implicate that this miRNA is under direct transcriptional control of the GR. Besides the precursors of miR-15b, miR-16 and miR-223 also pre-miR-19a and pre-miR-92-1 are regulated by GCs, but this regulation was not detected at the mature level, possibly due to a long half life and stability of the miRNA. Alternatively RNA editing of the dsRNA of the miRNAs, as described for miR-142 and miR-223

[13], could also prevent its detection at the mature level. To exclude that technical properties of the Ambion system (e.g. badly designed probes) led to the lacking regulation of mature forms of miR-19a and miR-92 RT-PCR or northern blot analyses would have to be performed.

Definition of pri-miRNAs based on genomic localization of the miRNAs led to the identification of potential primary transcripts of 328 miRNAs. Of these 169 are detectable on the Affymetrix Exon array, making the Exon array an instrument for the simultaneous measurement of pre- and pri-miRNAs and other coding and non-coding transcripts (including potential target genes of miRNAs). Although correlation of regulation values (M values) between most pre- and pri-miRNA pairs was poor, the pri-miRNAs of miR-223 (ENSESTE00000613173), miR-15b, miR-16 (both SMC4), miR-19a and miR-92-1 (both C13orf25) exhibited the same increasing regulation with treatment time as their pre-miRNAs, resulting in high correlation values ($R^2 = 0.977$, $R^2 = 0.908$, $R^2 = 0.994$, $R^2 = 0.935$ and $R^2 = 0.865$ respectively).

Prediction of the target genes and GO analysis on these genes revealed the potential function of the GC regulated miRNAs. How an identified processes is affected by the miRNAs, if it is activated or repressed, depends on the balance between activators and inhibitors regulated by the miRNAs, and on the extend of the target's repression by the miRNA, which is highly sequence specific and depends most probably also on the number of miRNA target sites in the 3' UTR as well as the sequence similarity of the miRNA and the binding site. Since miRNAs repress genes by preventing their neo-genesis, also the half life of the respective protein influences the impact of miRNA mediated repression. Post-transcriptional repression of genes with a high turnover would have more immediate effects on the biological process than regulation of genes with a long half life.

miRNAs regulate their target genes either by translational repression or by mRNA cleavage. The former is solely detectable on protein level, the latter also on mRNA level. Repression of target genes of the GC regulated miRNAs on mRNA level however is not stronger than expected by chance. Thus the 3 miRNAs most probably regulate their target genes by translational repression rather than by mRNA cleavage.

213 of the 1090 miRNA target genes are also significantly regulated after 24 hours GC treatment, thus are either direct or indirect targets of the GR. Therefore the GR and its regulated miRNAs could constitute direct or indirect feed forward loops (FFL) regulating the same target genes. Functional implications of such FFLs however are still elusive [34].

Identification of the biological processes *cell cycle* and *regulation of myeloid cell differentiation* by the GO analysis is in concordance with previous findings [37, 38]. The enriched process *regulation of transcription, DNA-dependent* and other transcription related processes on the other hand could represent a not yet known, regulatory feedback loop. In this model the GR could dampen its own transcriptional potential with the induction of the miRNAs, that subsequently repress factors needed for transcription. On the other hand miRNA mediated regulation of genes involved in transcription could promote GR mediated transcription, if mostly inhibitors were repressed.

Also *apoptotic mitochondrial changes* and *cell death* are among the enriched GO terms implicating that miR-15b, miR-16 and miR-223 could in fact play an important role in the GC induced apoptosis. These findings are also reflected in published data showing that the chromosomal region 13q14 hosting miR-15a and miR-16-1, which is frequently deleted in chronic lymphocytic leukemia (CLL), inversely correlates with high levels of the anti-apoptotic BCL2 gene [22] and that overexpression of miR-15a and miR-16-1 induces apoptosis in cell lines by strong reduction of BCL2 protein levels after 48h [22, 39]. Fulci et al in contrast did not find highly expressed BCL2 in CLL patients lacking miR-15a and miR-16, however the researchers did not investigate expression of miR-15b which also has the ability to control BCL2 expression. The GC regulated miR-15b has high sequence similarity with miR-15a and thus most likely also promotes the same pro-apoptotic effects. The mature miR-16, also regulated by GCs, can be processed from two distinct precursors, miR-16-1, located within a miRNA cluster with miR-15a on chromosome 13 and miR-16-2, which resides, together with miR-15b, in the polycistronic cluster on chromosome 3, located within an intron of the SMC4 gene. In ALL cells only the polycistronic cluster on chromosome 3 is regulated by GCs, giving raise to mature miR-15b and miR-16. Regulation of the miRNAs miR-15a and miR-16-1 was not detected on pri- pre- and mature miRNA levels. The myeloid specific differentiation signal mediated by miR-223 might have adverse effects in the lymphoid lineage and could contribute also to pro-apoptotic

signaling.

Downregulation of miR-19a miR-92-1, both part of the miR-17-92 cluster on chromosome 13, was detected at the pri- and pre-miRNA level, but GC mediated downregulation was not observed on mature level. Nevertheless these miRNAs have been characterized as anti-apoptotic, proliferative miRNAs [45, 42]. Expression of this cluster is tightly regulated by the proto-oncogene Myc [46] and their overexpression led to accelerated tumor development in a mouse B-cell lymphoma model [28], whereas repression of the cluster has shown to induce apoptosis in lung cancer cells overexpressing miR-17-92 [47].

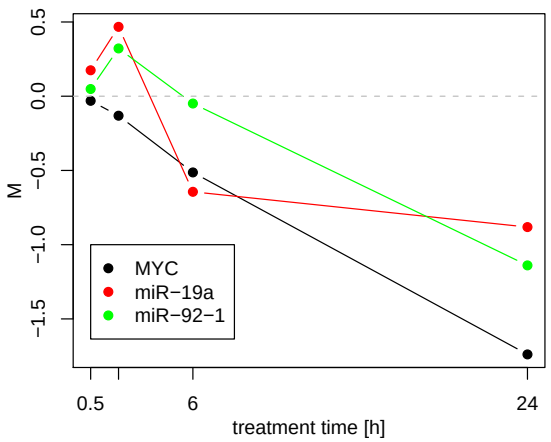


Figure 9: Correlation between MYC and miR-19a and miR-92-1. Expression of miR-19a miR-92-1 cluster is tightly regulated by MYC [46]. Thus the GC mediated repression of MYC results in a downregulation of miR-19a and miR-92-1.

MYC is repressed by GCs in CCRF-CEM-C7H2 cells and subsequently also pri- and pre-miR-19a and pre-miR-92-1 levels decrease (see figure 9). This finding is in line with the observation that expression of miR-17-92 cluster is controlled by MYC. Downregulation of the proproliferative/antiapoptotic miR-19a and miR-92-1 would perfectly fit into the model of GC regulated miRNAs facilitating or accelerating GC induced apoptosis.

Taken together miRNAs might play a critical role in the GC induced apoptosis i.e. by facilitating or accelerating apoptosis by repressing anti-apoptotic genes like BCL2, Bcl-XL or Bcl-W. Figure 8 summarizes these results and presents a potential model for the contribution of miRNAs to GC induced apoptosis.

Material and Methods

Identification of Exon probes targeting pre-miRNAs

In order to identify oligo probes on the Exon array that allow to detect miRNA precursors the ∼5 million 25nt long oligo probes present on the Exon chip were mapped to the pre-miRNA sequences from the Sanger miRBase (version 10.0 [48]). Furthermore the chromosomal location of each probe was determined using exonerate (*http://www.ebi.ac.uk/∼guy/exonerate/*) and the complete genomic DNA sequences from Ensembl. These informations were inserted into a MySQL database and served for the generation of the chromosomal location plots (figure 3) and for the preprocessing of the Exon chips (for the summarization of oligo probes to one intensity value per pre-miRNA). In total 1108 oligonucleotide probes targeting 259 distinct human pre-miRNA sequences (on average 4 probes per pre-miRNA) were identified.

Definition of pri-miRNAs

Only a handful pri-miRNAs have been defined and experimentally validated so far. In order to use the Exon GeneChip for the detection of pri-miRNAs it is crucial to identify possible pri-miRNAs of all known miRNAs. miRNAs are largely subdivided in intronic and exonic miRNAs depending on their genomic location. Intronic miRNAs are embedded in introns of protein- or non-coding transcripts, whereas exonic miRNAs are located within exons of their own primary transcripts (i.e. the non-coding H19 gene, which is the pri-miRNA of miR-675 [25]). Since frequent co-expression of miRNAs with their host genes has been observed [49], host genes are commonly defined as pri-miRNAs for intronic miRNAs.

pri-miRNAs were identified based on information from the miRGen database (version 3 [26]) and Ensembles Biomart database (*http://www.biomart.org*). From the 472 miRNAs in the miRGen database, 290 are a lo-

cated within introns, exons or UTRs of transcripts of known genes, Refseq transcripts or predicted GenScan genes. Further 113 Ensembl transcripts have been identified employing Bioconductors `biomaRt` package [50] as interface to the Ensembl database. All genomic features close to the genomic location of the 528 miRNAs from miRBase (version 10.0) were extracted from the Biomart database and potential pri-miRNAs were identified by comparing the genomic location of the exons of these transcripts to the genomic location of the miRNAs (thus identifying transcripts with exonic, intronic and partial exonic miRNAs). Union of the two lists resulted in possible pri-miRNAs for 321 miRNAs. The pri-miRNA of miR-223 was described previously [43] but was not identified by neither of the two approaches above. Thus the pri-miRNA and corresponding Exon probesets were identified and added manually utilizing the UCSC genome browser. The predicted pri-miRNA (GENSCAN00000035806) for the miRNA cluster on chromosome X consisting of miRNAs miR-363, miR-92-2, miR-19b-2, miR-20b, miR-18b and miR-106a was defined similarly.

For each of the pri-miRNA transcripts *crosshyb_type 1* probesets (all probes in the probeset perfectly match only one transcribed genomic locus) were determined utilizing an annotation database based on Affymetrix NetAffx annotation version 22. In total 306 transcripts corresponding to primary transcripts of 201 out of 328 miRNAs are detectable on the Exon array. Only a few probesets could be matched to GenScan based predicted pri-miRNAs. Approximately 50% of known miRNAs are found in clusters and are transcribed as polycistronic primary transcripts [8], thus the 306 detectable transcripts on the Exon array represent alternative splice variants of 169 unique pri-miRNA-genes.

The R functions for the definition of the pri-miRNAs and the generation of the *chromosomal location plots* (e.g. figures 3, 4) are available at *http://madb.i-med.ac.at/R/miRNA-exon-plot.R*

Microarray data preprocessing and analysis

Ambion microarrays were scanned with a GenePix 4000B scanner (Axon Instruments, GenePix software version 5.0). Further analysis was performed in R (version 2.5 [51]) utilizing Bioconductor (version 2.0 [52]). Assuming identical optical background per dye-swap-pair (since the dye-swap pair was hybridized onto the same glass surface), the measured intensities of the same samples, but labeled with different dyes, were averaged to adjust for the dye-bias, inherent to two-color microarrays

For the detection of differentially expressed pre- and pri-miRNAs the same set of Exon arrays was analyzed. The two analyses were performed analogously, but differ substantially in the preprocessing of the raw data. To adjust signal intensities across chips the Exon array raw data was quantile normalized [53]. For the pre-miRNA analysis all probes targeting the same pre-miRNAs were summarized to one measurement per pre-miRNA utilizing RMA [54]. For the pri-miRNA analysis summarization was performed on all probes mapping to the same pri-miRNA transcript. Preprocessings resulted in two datasets consisting of 259 and 306 expression values of pre-miRNAs and pri-miRNA transcripts respectively.

p-values assessing significance of differential expression between GC treated and control samples for each timepoint were calculated using the moderated t-test [55]. The moderated t-test is especially suited for small sized groups (e.g. 3 replicates per group). With only three measurements per group the estimate of the standard error of the effect size is not stable and some genes get small p-values only because, by chance, the denominator of the t-statistic (the standard error) was very small. The moderated t-test accounts for this *sampling error* by borrowing strength across all genes to get a more stable estimate of gene-specific variance, similar to the method implemented in SAM [56]. p-values were further adjusted for multiple hypothesis testing employing the method proposed by Benjamin and Hochberg for a strong control of the false discovery rate (FDR) [57]. pre-miRNAs and pri-miRNAs are called significantly regulated if their Benjamini and Hochberg adjusted p-value $p_{BH} < 0.05$ (false discovery rate smaller 5%). In order to avoid multiple testing of the same pri-miRNA-gene by its various transcripts, the splice variant with the highest variance across all samples per timepoint (measured by the interquartile range (IQR)), and thus the highest information content, was used for the detection of differentially expressed pri-miRNAs.

miRNA target prediction and correlation analysis

miRNAs bind to specific sites in the 3' UTR of their target genes and post-transcriptionally repress their trans-

lation by a variety of mechanisms [11]. Definition of the miRNAs' target genes is crucial for the assessment of their potential functional role. Since only a handful miRNA targets have been identified and validated experimentally, target predictions base solely on computational methods. Current methods like PicTar [18], TargetScanS [19] and miRanda [17] make use of the, experimentally veryfied, miRNA:target interaction/recognition model. Most miRNA target sites are characterized by perfect base pairing in the seed region, defined as the first $\sim$ 7nt starting from the first or second nucleotide from the miRNA's 5' end. Alternatively, 3' compensatory sites have been described, that do not exhibit perfect base pairing in the seed region, but, to compensate for this, have extensive base pairing in the 3' end of the miRNA. All prediction algorithms further base on conservation of the target sites across at least two species to reduce the large number of false positive findings that are characteristic for the search of short stretches of perfect base pairings. For the definition of miRNA targets three popular programs were used: PicTar, TargetScanS and miRanda (reviewed in [58]).

PicTar [18] requires perfect matching at the seed region defined as the first 7nt from the first or second base from the 5' end of the miRNA. For all sites binding energies are calculated and the resulting free energy of the entire miRNA:mRNA duplex has to be below a cutoff value. Target sites not conserved across several species are filtered out as false positives.

TargetScanS [19] also requires perfect base pairing in the seed region (6nt from base 2-7 from the miRNAs 5' end). Furthermore the target sites must exhibit conservation of this seed across 5 genomes (human, mouse, rat, dog and chicken).

miRanda [17] calculates scores for miRNA:mRNA duplexes by performing a dynamic programming local alignment of the miRNA sequence with the mRNAs 3' UTR. The scores for the nucleotides 2-8 from the 5' end of the miRNA (the seed) are counted twice, thus detecting perfect seed matching targets. Additionally miRanda identifies 3' compensatory sites if extensive base pairing occurs at the 3' end of the miRNA. Additional to the alignment scores binding energies are calculated. To reduce false positive findings miRanda requires conservation across two or more species. Furthermore the positions of target sites in two species have to be within ± 10 residues in aligned 3' UTRs.

Targets for the GC regulated miRNAs were defined by intersection of the results from PicTar and TargetScanS (both focusing on perfect seed matching sites) followed by an union with the predictions of miRanda (which detects also 3' compensatory sites) as proposed by Sethupathy et al. [20]. The analysis bases on the target predictions provided by the miRGen web ressource [26]. The downloaded files (miRGen version 3) were processed in R and predicted targets for the GC regulated miRNAs were identified. Since some prediction algorithms return Ensembl Gene IDs of the predicted miRNA targets, other RefSeq gene IDs, all IDs were mapped to EntrezGene using facilities provided by Bioconductor's `biomaRt` package. This mapping yielded 862 target genes for miR-15b, 819 for miR-16 and 294 for miR-223. Some genes are predicted targets of two or more miRNAs, thus in total 1208 target genes were predicted for the GC regulated miRNAs. For the resulting list of EntrezGenes all probesets/transcripts detectable on the Affymetrix Exon arrays were identified and expression values for these probesets were extracted from the 24 Affymetrix Exon arrays already employed in the analysis of differentially expressed pre- and primiRNAs.

To avoid duplicate measures of the same target gene by multiple transcripts/probesets on the Exon array, the transcript either predicted by the algorithms, or, if this is not detectable on the Exon array, the trabscript with the highest variance across M values was selected for each target gene.

To assess significance for the finding that the M values of the target genes exhibit a increasing downregulation over time, the difference of the M values after 2 and 24 hours of the target genes (average $M_{2h} - M_{24h} = -0.035$, p-value=0.0000836) was compared to those of a background gene set. Under the NULL hypothesis that any random gene set shows the same downregulation, the observed t-statistic t_{obs} (paired t-test of the M values after 24 hours against those after 2 hours treatment) was compared to 10000 expected t-statistics t_{exp}, calculated for 10000 random gene sets (randomly selected genes among all genes detectable on the Exon array (except the miRNA target genes), the gene set size was kept equal to the number of target genes).

GO analysis

A Gene Ontology (GO [35]) analysis aims to support the biological interpretation of any list of genes by iden-

tifying e.g. biological processes in which the genes of the gene set are over-represented compared to a background gene set (significance calculated with Fisher's exact test). A common problem in a GO analysis is, that directly related GO terms (i.e. parent-child nodes) often have a considerable overlap of genes, thus often parent GO terms are called significant, because most of its child nodes are enriched. Due to the hierarchical structure of the gene ontology parent nodes inherit all genes annotated at their, more specific, child nodes. To alleviate this problem a method similar to that of Alexa et al. [59] was used. This method removes genes annotated to more general terms, if these genes are annotated to more specific and enriched child terms. Additionally, to improve the quality of the associations between genes and GO terms, all annotations based solely on computational sequence similarity searches were excluded from the analysis (*IEA* (inferred from electronic annotation) GO evidence code).

The GO analysis in this analysis aimed to identify biological processes (GO terms of the biological process GO) that are enriched among the predicted target genes of the GC regulated miRNAs miR-15b, miR-16 and miR-223 compared to a background set of genes (further on referred as *gene universe*), defined as the set of genes predicted as target of any miRNA by one of the three prediction algorithms PicTar, TargetScanS and miRanda. The GO analysis was performed in R (version 2.5.1) using Bioconductors `humanLLMappings` package for the association of genes to GO terms and the `GOstats` package [60] to identify over-represented GO terms for the target genes. From the 10119 genes in the gene universe 4359 are associated to one or more GO terms, from the 1208 target genes 912.

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References

[1] S Schmidt, J Rainer, S Riml, C Ploner, S Jesacher, C Achmuller, E Presul, S Skvortsov, R Crazzolara, M Fiegl, T Raivio, O A Janne, S Geley, B Meister, and R Kofler. Identification of glucocorticoid-response genes in children with acute lymphoblastic leukemia. *Blood*, 107(5):2061–2069, Mar 2006. Comparative Study.

[2] S Schmidt, J Rainer, C Ploner, E Presul, S Riml, and R Kofler. Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance. *Cell Death Differ*, 11 Suppl 1:45–55, Jul 2004.

[3] C Ploner, S Schmidt, E Presul, K Renner, K Schrocksnadel, J Rainer, S Riml, and R Kofler. Glucocorticoid-induced apoptosis and glucocorticoid resistance in acute lymphoblastic leukemia. *J Steroid Biochem Mol Biol*, 93(2-5):153–160, Feb 2005.

[4] I Herr, N Gassler, H Friess, and M W Buchler. Regulation of differential pro- and anti-apoptotic signaling by glucocorticoids. *Apoptosis*, 12(2):271–291, Feb 2007.

[5] Tissing WJ, Den Boer ML, Meijerink JP, Menezes RX, Swagemakers S, van der Spek PJ, Sallan SE, Armstrong SA, and Pieters R. Genome-wide identification of prednisolone-responsive genes in acute lymphoblastic leukemia cells. *Blood*, Jan 2007. JOURNAL ARTICLE.

[6] G A Calin and C M Croce. MicroRNA-cancer connection: the beginning of a new tale. *Cancer Res*, 66(15):7390–7394, Aug 2006.

[7] R Garzon, M Fabbri, A Cimmino, G A Calin, and C M Croce. MicroRNA expression and function in cancer. *Trends Mol Med*, 12(12):580–587, Dec 2006.

[8] V N Kim and Jin-Wu Nam. Genomics of microRNA. *Trends Genet*, 22(3):165–173, Mar 2006.

[9] B R Cullen. Transcription and processing of human microRNA precursors. *Mol Cell*, 16(6):861–865, Dec 2004.

[10] X Cai, C H Hagedorn, and B R Cullen. Human microRNAs are processed from capped, polyadenylated

transcripts that can also function as mRNAs. *RNA*, 10(12):1957–1966, Dec 2004.

[11] R S Pillai, S N Bhattacharyya, and W Filipowicz. Repression of protein synthesis by miRNAs: how many mechanisms? *Trends Cell Biol*, 17(3):118–126, Mar 2007.

[12] D P Bartel. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, 116(2):281–297, Jan 2004.

[13] W Yang, T P Chendrimada, Q Wang, M Higuchi, P H Seeburg, R Shiekhattar, and K Nishikura. Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. *Nat Struct Mol Biol*, 13(1):13–21, Jan 2006.

[14] J M Thomson, M Newman, J S Parker, E M Morin-Kensicki, T Wright, and S M Hammond. Extensive post-transcriptional regulation of microRNAs and its implications for cancer. *Genes Dev*, 20(16):2202–2207, Aug 2006.

[15] G Obernosterer, P J F Leuschner, M Alenius, and J Martinez. Post-transcriptional regulation of microRNA expression. *RNA*, 12(7):1161–1167, Jul 2006.

[16] Y Kawahara, B Zinshteyn, P Sethupathy, H Iizasa, A G Hatzigeorgiou, and K Nishikura. Redirection of silencing targets by adenosine-to-inosine editing of miRNAs. *Science*, 315(5815):1137–1140, Feb 2007.

[17] B John, A J Enright, A Aravin, T Tuschl, C Sander, and D S Marks. Human MicroRNA targets. *PLoS Biol*, 2(11):e363, Nov 2004.

[18] A Krek, D Grun, M N Poy, R Wolf, L Rosenberg, E J Epstein, P MacMenamin, I da Piedade, K C Gunsalus, M Stoffel, and N Rajewsky. Combinatorial microRNA target predictions. *Nat Genet*, 37(5):495–500, May 2005.

[19] B P Lewis, C B Burge, and D P Bartel. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, 120(1):15–20, Jan 2005. Letter.

[20] P Sethupathy, M Megraw, and A G Hatzigeorgiou. A guide through present computational approaches for the identification of mammalian microRNA targets. *Nat Methods*, 3(11):881–886, Nov 2006. Comparative Study.

[21] W Wu, M Sun, G-M Zou, and J Chen. MicroRNA and cancer: Current status and prospective. *Int J Cancer*, 120(5):953–960, Mar 2007.

[22] A Cimmino, G A Calin, M Fabbri, M V Iorio, M Ferracin, M Shimizu, S E Wojcik, R I Aqeilan, S Zupo, M Dono, L Rassenti, H Alder, S Volinia, C G Liu, T J Kipps, M Negrini, and C M Croce. mir-15 and mir-16 induce apoptosis by targeting bcl2. *Proc Natl Acad Sci U S A*, 102(39):13944–13949, Sep 2005.

[23] E M Strasser-Wozak, R Hattmannstorfer, M Hala, B L Hartmann, M Fiegl, S Geley, and R Kofler. Splice site mutation in the glucocorticoid receptor gene causes resistance to glucocorticoid-induced apoptosis in a human acute leukemic cell line. *Cancer Res*, 55(2):348–353, Jan 1995.

[24] J Rainer, S Jesacher, A Ploner, H Niederegger, C Ploner, and R Kofler. Glucocorticoids regulate microRNAs-223 and 15b 16-2 in human acute lymphoblastic leukemia cells. 2007. submitted.

[25] X Cai and B R Cullen. The imprinted H19 noncoding RNA is a primary microRNA precursor. *RNA*, 13(3):313–316, Mar 2007.

[26] M Megraw, P Sethupathy, B Corda, and A G Hatzigeorgiou. miRGen: a database for the study of animal microRNA genomic organization and function. *Nucleic Acids Res*, 35(Database issue):149–155, Jan 2007.

[27] Y Altuvia, P Landgraf, G Lithwick, N Elefant, S Pfeffer, A Aravin, M J Brownstein, T Tuschl, and H Margalit. Clustering and conservation patterns of human micrornas. *Nucleic Acids Res*, 33(8):2697–2706, 2005.

[28] L He, J M Thomson, M T Hemann, E Hernando-Monge, D Mu, S Goodson, S Powers, C Cordon-Cardo, S W Lowe, G J Hannon, and S M Hammond. A microrna polycistron as a potential human oncogene. *Nature*, 435(7043):828–833, Jun 2005.

[29] F Fazi, A Rosa, A Fatica, V Gelmetti, M L De Marchis, C Nervi, and I Bozzoni. A minicircuitry comprised of microrna-223 and transcription factors nfi-a and c/ebpalpha regulates human granulopoiesis. *Cell*, 123(5):819–831, Dec 2005.

[30] J Brennecke, A Stark, R B Russell, and S M Cohen. Principles of microRNA-target recognition. *PLoS Biol*, 3(3):e85, Mar 2005.

[31] P Sood, A Krek, M Zavolan, G Macino, and N Rajewsky. Cell-type-specific signatures of microRNAs on target mRNA expression. *Proc Natl Acad Sci U S A*, 103(8):2746–2751, Feb 2006.

[32] L P Lim, N C Lau, P Garrett-Engele, A Grimson, J M Schelter, J Castle, D P Bartel, P S Linsley, and J M Johnson. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*, 433(7027):769–773, Feb 2005.

[33] N Rajewsky. microrna target predictions in animals. *Nat Genet*, 38 Suppl:8–13, Jun 2006.

[34] R Shalgi, D Lieber, M Oren, and Y Pilpel. Global and local architecture of the mammalian microrna-transcription factor regulatory network. *PLoS Comput Biol*, 3(7), Jul 2007.

[35] M Ashburner, C A Ball, J A Blake, D Botstein, H Butler, J M Cherry, A P Davis, K Dolinski, S S Dwight, J T Eppig, M A Harris, D P Hill, L Issel-Tarver, A Kasarskis, S Lewis, J C Matese, J E Richardson, M Ringwald, G M Rubin, and G Sherlock. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet*, 25(1):25–29, May 2000.

[36] J Lu, G Getz, E A Miska, E Alvarez-Saavedra, J Lamb, D Peck, A Sweet-Cordero, B L Ebert, R H Mak, A A Ferrando, J R Downing, T Jacks, H R Horvitz, and T R Golub. Microrna expression profiles classify human cancers. *Nature*, 435(7043):834–838, Jun 2005.

[37] C Z Chen, L Li, H F Lodish, and D P Bartel. Micrornas modulate hematopoietic lineage differentiation. *Science*, 303(5654):83–86, Jan 2004.

[38] P S Linsley, J Schelter, J Burchard, M Kibukawa, M M Martin, S R Bartz, J M Johnson, J M Cummins, C K Raymond, H Dai, N Chau, M Cleary, A L Jackson, M Carleton, and L Lim. Transcripts targeted by the microrna-16 family cooperatively regulate cell cycle progression. *Mol Cell Biol*, 27(6):2240–2252, Mar 2007.

[39] R Garzon, F Pichiorri, T Palumbo, M Visentini, R Aqeilan, A Cimmino, H Wang, H Sun, S Volinia, H Alder, G A Calin, C G Liu, M Andreeff, and C M Croce. Microrna gene expression during retinoic acid-induced differentiation of human acute promyelocytic leukemia. *Oncogene*, 26(28):4148–4157, Jun 2007.

[40] C Ploner, J Rainer, H Niederegger, M Eduardoff, A Villunger, S Geley, and R Kofler. The BCL2 rheostat in glucocorticoid-induced apoptosis of acute lymphoblastic leukemia. 2007. submitted.

[41] K Renner, M J Ausserlechner, and R Kofler. A conceptual view on glucocorticoid-lnduced apoptosis, cell cycle arrest and glucocorticoid resistance in lymphoblastic leukemia. *Curr Mol Med*, 3(8):707–717, Dec 2003.

[42] M Jovanovic and M O Hengartner. mirnas and apoptosis: Rnas to die for. *Oncogene*, 25(46):6176–6187, Oct 2006.

[43] T Fukao, Y Fukuda, K Kiga, J Sharif, K Hino, Y Enomoto, A Kawamura, K Nakamura, T Takeuchi, and M Tanabe. An evolutionarily conserved mechanism for microRNA-223 expression revealed by microRNA gene profiling. *Cell*, 129(3):617–631, May 2007.

[44] J Kluiver, B J Kroesen, S Poppema, and A van den Berg. The role of micrornas in normal hematopoiesis and hematopoietic malignancies. *Leukemia*, 20(11):1931–1936, Nov 2006.

[45] H W Hwang and J T Mendell. Micrornas in cell proliferation, cell death, and tumorigenesis. *Br J Cancer*, 96 Suppl:40–44, 2007.

[46] K A O'Donnell, E A Wentzel, K I Zeller, C V Dang, and J T Mendell. c-myc-regulated micrornas modulate e2f1 expression. *Nature*, 435(7043):839–843, Jun 2005.

[47] H Matsubara, T Takeuchi, E Nishikawa, K Yanagisawa, Y Hayashita, H Ebi, H Yamada, M Suzuki, M Nagino, Y Nimura, H Osada, and T Takahashi. Apoptosis induction by antisense oligonucleotides against mir-17-5p and mir-20a in lung cancers overexpressing mir-17-92. *Oncogene*, Mar 2007.

[48] S Griffiths-Jones, R J Grocock, S van Dongen, A Bateman, and A J Enright. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res*, 34(Database issue):140–144, Jan 2006.

[49] S Baskerville and D P Bartel. Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. *RNA*, 11(3):241–247, Mar 2005.

[50] S Durinck, Y Moreau, A Kasprzyk, S Davis, B De Moor, A Brazma, and W Huber. BioMart and Bioconductor: a powerful link between biological databases and microarray data analysis. *Bioinformatics*, 21(16):3439–3440, Aug 2005.

[51] R Development Core Team. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria, 2007. ISBN 3-900051-07-0.

[52] R C Gentleman, V J Carey, D M Bates, B Bolstad, M Dettling, S Dudoit, B Ellis, L Gautier, Y Ge, J Gentry, K Hornik, T Hothorn, W Huber, S Iacus, R Irizarry, F Leisch, C Li, M Maechler, A J Rossini, G Sawitzki, C Smith, G Smyth, L Tierney, J Y H Yang, and J Zhang. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol*, 5(10):R80, 2004.

[53] B M Bolstad, R A Irizarry, M Astrand, and T P Speed. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics*, 19(2):185–193, Jan 2003. Comparative Study.

[54] R A Irizarry, B M Bolstad, F Collin, L M Cope, B Hobbs, and T P Speed. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res*, 31(4):e15, Feb 2003.

[55] G K Smyth. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol*, 3:Article3, 2004.

[56] V G Tusher, R Tibshirani, and G Chu. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A*, 98(9):5116–5121, Apr 2001.

[57] S Dudoit, J P Shaffer, and J C Boldrick. Multiple Hypothesis Testing in Microarray Experiments. *Stat Sci*, 18:71–103, 2003.

[58] I Ioshikhes, S Roy, and C K Sen. Algorithms for mapping of mRNA targets for microRNA. *DNA Cell Biol*, 26(4):265–272, Apr 2007.

[59] A Alexa, J Rahnenfuhrer, and T Lengauer. Improved scoring of functional groups from gene expression data by decorrelating GO graph structure. *Bioinformatics*, 22(13):1600–1607, Jul 2006.

[60] S Falcon and R Gentleman. Using GOstats to test gene lists for GO term association. *Bioinformatics*, 23(2):257–258, Jan 2007.

Glucocorticoids regulate microRNAs-223 and 15b~16-2 in human acute lymphoblastic leukemia cells

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[3]Abbreviations used in this paper: ALL, acute lymphoblastic leukemia; GC, glucocorticoid; GR glucocorticoid receptor; miRNA, microRNA;

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Abstract

Glucocorticoids (GC) have a major impact on the biology of normal and malignant cells of the lymphoid lineage. This includes induction of apoptosis which is exploited in the therapy of acute lymphoblastic leukemia (ALL) and related lymphoid malignancies. MicroRNAs (miRNAs) are ~22 nucleotide RNA molecules that control essential biological function including proliferation, differentiation and apoptosis. They derive from polymerase-II transcripts but whether GCs regulate miRNA-encoding transcription units is not known. We investigated miRNA expression and regulation in GC-treated CCRF-CEM T-ALL cells by expression profiling, real time RT-PCR and northern blotting. Three miRNAs, i.e., miR-15b, miR-16-2 and miR-223, were induced in these ALL cells both as mature miRNAs and at the level of their putative precursors (SMC4 for miR-15b~16-2, and LOC389865 for miR-223). Since miR-223 is involved in hematopoietic differentiation and miRNAs of the 15/16 family affect apoptosis, their induction might contribute to the effects of GC on lymphoid lineage cells.

Introduction

Glucocorticoids (GCs) have pronounced effects on metabolism, differentiation, proliferation and cell survival in many tissues. In the lymphoid system, they affect cell cycle progression, influence immunoglobulin and lymphokine production and, most notably, induce apoptosis in immature lymphoblasts. The latter has been implicated in the generation of the immune repertoire and the regulation of immune responses (1-3), and is clinically used in the treatment of childhood acute lymphoblastic leukemia (ALL) and other lymphoid malignancies (4). GCs mediate their effects via the GC receptor (GR), a ligand-activated transcription factor of the nuclear receptor super-family that resides in the cytoplasm and, upon ligand binding, translocates into the nucleus, where it modulates gene expression via binding to specific DNA

response elements or by protein-protein interactions with other transcription factors (5). A large number of protein-encoding genes has been identified that are regulated by GCs in lymphoid lineage cells in experimental systems (6) and related clinical samples (7,8), but the genes responsible for cell death induction and other effects of GCs on the immune system are not well understood (for recent reviews see)(2,6,9,10).

Recently, a novel class of tiny non-coding RNAs, referred to as microRNAs (miRNAs), has been identified that regulates the stability or translational efficiency of target mRNAs (for recent reviews see (11-14)). They are transcribed as long primary transcripts (pri-miRNAs) by RNA polymerase II (15,16), but only a few pri-miRNAs have been fully defined to date. miRNAs are either embedded in introns of protein-coding or non-coding transcripts (intronic miRNAs) or constitute independent non-coding RNAs transcribed from their own promoters (exonic miRNAs) (17). Frequently, 2 or more miRNAs are clustered in polycistronic transcripts. Subsequent to transcription, pri-miRNAs are processed by the Drosha, a member of the RNase III family of enzymes, to generate ~70 nucleotide precursors called pre-miRNAs. Pre-miRNAs are exported to the cytoplasm by Exportin-5 where they are cleaved by Dicer, another RNase III enzyme, to generate ~22 base pair duplex intermediates. The duplexes enter effector complexes called “miRNPs” (miRNA-containing ribonucleoprotein particles), “mirgonaute”, or more generally, miRISC (miRNA-containing RNA-induced silencing complex), where they are rapidly converted into single stranded “mature miRNAs” (11). Thus, based on their biogenesis, miRNAs can be detected as long polyadenylated pri-miRNAs, ~70 nucleotide pre-miRNAs and ~22 nucleotide mature miRNAs. Moreover, mature mRNAs (without their intronic miRNA sequences) have been detected in the cytoplasm (16), often in strong correlation with the expression of the intronic miRNA (13,17).

Despite advances in our understanding of miRNA biology, little is known about the transcriptional regulation of miRNAs and only a few mammalian transcription factors that regulate miRNAs have been identified including c-myc which regulates the "oncogenic" miRNA cluster miR-17-5p~18a~19a~20a~19b-1~92-1 (18), or PU.1 and C/EBPβ which controls the hematopoietic miRNA, miR-223 (19). Whether expression of miRNAs is subject to regulation by GC and the GR has not been addressed. Since miRNAs might contribute to the anti-leukemic effects of GC, we initiated a search for GC-regulated miRNAs in CCRF-CEM cells, a widely studied *in vitro* model for GC-induced leukemia apoptosis.

Materials and methods

Cell lines

GC-sensitive T-ALL CCRF-CEM-C7H2 (20) were cultured in RPMI 1640 supplemented with 10% fetal calf serum and 2mM L-glutamine at 37ºC, 5% carbon-dioxide and saturated humidity. The cells were free of mycoplasma infection and their authenticity was verified by DNA fingerprinting as detailed previously (21).

RNA preparation and microRNA expression profiling

Total RNA was prepared from 3 biological replicates of CEM-C7H2 cells treated with 100nM dexamethasone (Sigma, Vienna, Austria) or 0.1% ethanol as carrier control for 2h, 6h, 12h and 24h using mirVana™ miRNA isolation kit (Ambion/ABI, Foster City, CA). The replicate RNAs were pooled and small RNAs containing mature miRNAs were isolated using the flashPAGE™ system, labeled with the mirVana™ miRNA labeling kit and hybridized to mirVana™ miRNA Bioarrays (version 1, detecting 320 human microRNAs) according to the provider´s protocols (http://www.ambion.com). In each case, dye swap experiments were performed (i.e., the control sample was labeled with Cy3, the treated sample with Cy5 and *vice versa*). Microarrays were

scanned with a GenePix 4000B scanner (Axon instruments, GenePix software version 5.0). Further analysis was performed in R (version 2.5) and Bioconductor (version 2.0) (22). Assuming identical optical background signal per array we adjusted for the dye bias by averaging the measured intensities for the 2 dyes for each sample. All features with an M-value (log2 fold change value) of >1 or < -1 (more than 2-fold up- or down-regulated) in both replicated spots on the microarray were considered GC-regulated.

Precursor microRNA identification on U133 and Exon arrays

Expression profiling data for CEM-C7H2 treated with 100nM dexamethasone or 0.1% ethanol as carrier control for 6h and 24h as singlicates (U133 Plus 2.0)(7) or biological triplicates (Exon 1.0)(23) have been published. For the U133 Plus 2.0 array we manually selected probe sets for the pri-miRNAs of the miRNAs found to be differentially expressed by the Ambion system using the miRGen database (24) and the UCSC Genome Browser. To identify probes on the Exon GeneChip that allow measurement of pri- and/or pre-miRNA expression we mapped the ~5 million Exon probes to pre-miRNA sequences from the miRBase database (version 9.2) (25) using exonerate (http://www.ebi.ac.uk/~guy/exonerate/). We identified 1019 oligonucleotide probes targeting 239 distinct human pre-miRNA sequences (on average 4 probes per pre-miRNA). U133 Plus 2.0 raw data files were preprocessed using GCRMA (26), Exon arrays were RMA (27) preprocessed (quantile normalization adjusting all probe signals on the array and summarization of the subset of probes targeting pre-miRNA sequences, resulting in one intensity measure per pre-miRNA). For the data on the Exon arrays, p-values for the significance of differential expression per time point were calculated using the moderated t-test (28) and adjusted for multiple hypothesis testing using the method of Benjamini and Hochberg (29).

Real time RT-PCR

Ten ng pooled total RNA (used for microRNA expression profiling) were reverse transcribed, diluted 1:15 and mature microRNAs were PCR-amplified using TaqMan® miRNA detection kits for hsa-miR-223, 15b, 16, 19b, 181a and, as a similarly highly expressed but unregulated control, hsa-miR-320 following the provider´s recommendations (Applied Biosystems, Foster City, CA). For hsa-miR-19b and 181a, we also used the mirVana™ qRT-PCR miRNA detection kit from Ambion (using 25ng total RNA).

Northern blotting

Twenty µg pooled total RNA (as used for microRNA expression profiling) were separated on 8% denaturing polyacrylamide gels (7 M urea, 1× TBE buffer), transferred onto nylon membranes and probed with 5′-[^{32}P] end-labeled antisense DNA probes for hsa-miR-15b, 16, 19b, 181a, 223 and 5.8 S rRNA as loading control (for sequences see supplementary Table 2) as described (30).

Results

GC regulate mature miRNA-15b, 16, and 223 in human T-ALL cells

To investigate whether GCs regulate miRNAs in human ALL cells, small RNAs from biological triplicates of CCRF-CEM-C7H2 cells cultured in the presence or absence of 100nM dexamethasone for 2h, 6h, and 24h were investigated for expression of 320 human microRNAs using the mirVana™ miRNA Bioarrays version 1 (see supplementary Table 1 for complete data). Five microRNAs were regulated more than 2-fold on both replicated spots on the array after dye swap normalization. miR-19b and 181a were regulated only at the early time points and not after 24h, induction of miR-223 was only detectable after 24h, and miR-15b and 16 showed increasing induction reaching significant levels after 24h (Table 1A).

We next attempted to reconfirm the regulations seen on the Ambion arrays by two additional approaches for detection of mature miRNAs, i.e., quantitative real time RT-PCR and northern blotting. Regulation of miR-19b and miR-181a could not be reconfirmed by either method even though we used 2 independent RT-PCR systems for detection (data not shown). In contrast, the inductions of miR-223, 15b and 16 were clearly seen by real time RT-PCR (Figure 1A) and northern blotting (Figure 1B). Therefore, we concluded that the expression of the mature forms of miR-223, 15b and 16 is induced by GC in this human T-ALL cell line.

GC regulation of miR223 and miR15b~16-2 occurs at the level of the primary transcript

Although regulation of miRNAs expression is often thought to occur at the level of the primary transcript, there is increasing evidence for post-transcriptional regulation (31,32). To closer define the level in biogenesis at which miR-223, 15b and 16 might be regulated, we exploited our expression profiles of GC-treated CEM-C7H2 cells performed on the Exon 1.0 and U133 plus 2.0 platforms (7,23). Neither platform detects mature miRNAs since they will be lost during target preparation due to their small size, but Exon arrays detect pri-miRNAs, and possibly pre-miRNAs because the RNA used for target labeling is randomly primed and the arrays contain probes detecting all known and predicted exons including numerous miRNAs. Targets for arrays of the U133-type, in contrast, are oligo-(dT)-primed, only polyadenylated pri-miRNAs can be detected on such arrays provided appropriate probe sets are present thereon. Given the currently accepted biogenesis pathway (miRNA containing gene → pri-miRNA → pre-miRNA → mature miRNA), regulations detected only on the Ambion array would suggest regulated action of Dicer, whereas additional regulation on the Exon but not the U133 array would reflect regulated Drosha activity, and regulation on all 3 platforms would argue for transcriptional control. As detailed in Table 1b, Exon probe sets for the pre-miRNA forms of miR-223 and 15b were significantly

induced after 24h. miR-16-2, which clusters with miR-15b in an intron of the SMC4 gene (17), showed a statistically insignificant tendency for induction (M=0.5). On the U133 array, the pri-miRNAs for miR-223 (LOC389865) and 15b~16-2 (SMC4) were clearly regulated already after 6h (Table 1C). Thus the combined data argue for a transcriptional control of both miRNA-containing transcription units.

In addition we found 3 further miRNAs on the Exon array that were GC-regulated (Table 1B and supplementary Table 3 for complete Exon data). One of them, miR-675, was clearly induced, however, because it was represented on the Exon array by a single probe only and was not regulated on the Ambion array, it was not followed up further in this study. Two others, miR-19a and 92-1 were significantly repressed after 24h (M= -0.8 and -1.2, pBH=0.032 and 0.0063, respectively). These miRNAs map to intron sequences of C13orf25, a putative protein-encoding transcript that was repressed on the U133 as well (Table 1C). However, C13orf25 is induced by c-myc (18) which is downregulated by GC in CCRF-CEM cells (33,34). Thus, the observed downregulation of miR-19a and 92-2 is, in all likelihood, secondary to GC repression of c-myc.

Discussion

This study demonstrates for the first time that GCs have the potential to regulate human miRNAs. In CCRF-CEM T-ALL cells, 3 miRNAs (the clustered miRNAs 15b~16-2, and miR-223) were regulated at the mature and precursor miRNA levels as detected by 5 independent techniques. Our data provide strong evidence that the observed regulations occur at the level of transcription, although whether the corresponding promoters are direct or indirect targets of the ligand-activated GR remains to be determined. miR-15b and 16-2 are classified as “intronic miRNAs” embedded in the SMC4 locus on chromosome 3 (17) that encodes the “structural maintenance of

chromosomes 4" protein. Whether the SMC4 promoter is directly regulated by GR is also unknown. The promoter for miR-223, an "exonic miRNA" of a non-coding transcription unit (17) termed LOC389865, has been identified in man and mouse and, at least in the latter, contains 2 GC response elements (GREs) (19). Functional analyses of GR dependency are lacking, and we are currently testing corresponding promoter constructs to address molecular details of the GC regulation of the above genes.

The functional consequences of the observed miRNA regulations is obviously a crucial question. miRNAs of the miR-15/16 family, i.e., 15a~16-1 and 15b~16-2 embedded in the DLEU2 locus on chromosome 13 and the SMC4 locus on chromosome 3 respectively (17), have been implicated in cell death/survival decisions, presumably by targeting BCL2 (35). Interestingly, BCL2 protein is indeed reduced in GC-treated CCRF-CEM-C7H2 cells with a relatively mild reduction of BCL2 mRNA (23). This observation would be well explained by the action of miRNAs which are known to act primarily by interfering with mRNA translation (14,36). Thus, induction of 15b and 16-2 might contribute to GC-induced apoptosis in this system. miR-223, which targets the transcription factor NFI-A (37), has been implicated in hematopoietic differentiation with particular emphasis on the myeloid lineage (19,38,39) and induction of a myeloid lineage specific miRNA in the lymphoid lineage might have adverse effects that could constitute a death signal.

Another important question is whether the regulations observed in this model system also occur in ALL patients during GC therapy, as is currently being investigated in our lab. A corresponding first analysis of U133 plus 2.0 arrays derived from 13 children (3 T-ALLs, 10 precursor B-ALLs) during systemic GC monotherapy (7) showed that the 15b~16-2 cluster was induced in 1 child with T-ALL, and pri-miR-223 was induced in about half of the children

(manuscript in preparation). Thus, the regulations we observed in an *in vitro* ALL model occur *in vivo* in GC-treated patients and might contribute to the therapeutic effects of GC in ALL.

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Disclosures

The authors have no financial conflict of interest.

References

1. Ashwell, J. D., F. W. Lu, and M. S. Vacchio. 2000. Glucocorticoids in T cell development and function. *Annu. Rev. Immunol.* 18:309-345.
2. Schaaf, M. J., and J. A. Cidlowski. 2002. Molecular mechanisms of glucocorticoid action and resistance. *J. Steroid Biochem. Mol. Biol.* 83:37-48.
3. Jondal, M., A. Pazirandeh, and S. Okret. 2001. A role for glucocorticoids in the thymus? *Trends Immunol.* 22:185-186.
4. Pui, C. H., M. V. Relling, and J. R. Downing. 2004. Acute lymphoblastic leukemia. *N. Engl. J. Med.* 350:1535-1548.
5. Laudet, V., and H. Gronemeyer. 2002. *The nuclear receptor facts book*. Academic Press, London, pp. 1.
6. Schmidt, S., J. Rainer, C. Ploner, E. Presul, S. Riml, and R. Kofler. 2004. Glucocorticoid-induced apoptosis and glucocorticoid resistance: Molecular mechanisms and clinical relevance. *Cell Death Differ.* 11 Suppl 1:S45-S55.
7. Schmidt, S., J. Rainer, S. Riml, C. Ploner, S. Jesacher, C. Achmüller, E. Presul, S. Skvortsov, R. Crazzolara, M. Fiegl, T. Raivio, O. A. Jänne, S. Geley, B. Meister, and R. Kofler. 2006. Identification of glucocorticoid response genes in children with acute lymphoblastic leukemia. *Blood* 107:2061-2069.
8. Tissing, W. J., M. L. den Boer, J. P. Meijerink, R. X. Menezes, S. Swagemakers, P. J. van der Spek, S. E. Sallan, S. A. Armstrong, and R. Pieters. 2007. Genome-wide identification of prednisolone-responsive genes in acute lymphoblastic leukemia cells. *Blood* 109:3929-3935.
9. Distelhorst, C. W. 2002. Recent insights into the mechanism of glucocorticosteroid-induced apoptosis. *Cell Death Differ.* 9:6-19.

10. Haarman, E. G., G. J. Kaspers, and A. J. Veerman. 2003. Glucocorticoid resistance in childhood leukaemia: mechanisms and modulation. *Br. J. Haematol.* 120:919-929.
11. Kim, V. N., and J. W. Nam. 2006. Genomics of microRNA. *Trends Genet.* 22:165-173.
12. Filipowicz, W. 2005. RNAi: the nuts and bolts of the RISC machine. *Cell* 122:17-20.
13. Cullen, B. R. 2004. Transcription and processing of human microRNA precursors. *Mol. Cell* 16:861-865.
14. Bartel, D. P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116:281-297.
15. Lee, Y., M. Kim, J. Han, K. H. Yeom, S. Lee, S. H. Baek, and V. N. Kim. 2004. MicroRNA genes are transcribed by RNA polymerase II. *EMBO J.* 23:4051-4060.
16. Cai, X., C. H. Hagedorn, and B. R. Cullen. 2004. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA.* 10:1957-1966.
17. Rodriguez, A., S. Griffiths-Jones, J. L. Ashurst, and A. Bradley. 2004. Identification of mammalian microRNA host genes and transcription units. *Genome Res.* 14:1902-1910.
18. O'Donnell, K. A., E. A. Wentzel, K. I. Zeller, C. V. Dang, and J. T. Mendell. 2005. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* 435:839-843.
19. Fukao, T., Y. Fukuda, K. Kiga, J. Sharif, K. Hino, Y. Enomoto, A. Kawamura, K. Nakamura, T. Takeuchi, and M. Tanabe. 2007. An evolutionarily conserved mechanism for microRNA-223 expression revealed by microRNA gene profiling. *Cell* 129:617-631.
20. Strasser-Wozak, E. M. C., R. Hattmannstorfer, M. Hála, B. L. Hartmann, M. Fiegl, S. Geley, and R. Kofler. 1995. Splice site mutation in the glucocorticoid receptor gene causes resistance to glucocorticoid-induced apoptosis in a human acute leukemic cell line. *Cancer Res.* 55:348-353.
21. Parson, W., R. Kirchebner, R. Mühlmann, K. Renner, A. Kofler, S. Schmidt, and R. Kofler. 2005. Cancer cell line identification by short tandem repeat profiling: power and limitations. *FASEB J.* 19:434-436.
22. Gentleman, R. C., V. J. Carey, D. M. Bates, B. Bolstad, M. Dettling, S. Dudoit, B. Ellis, L. Gautier, Y. Ge, J. Gentry, K. Hornik, T. Hothorn, W. Huber, S. Iacus, R. Irizarry, F. Leisch, C. Li, M. Maechler, A. J. Rossini, G. Sawitzki, C. Smith, G. Smyth, L. Tierney, J. Y. Yang, and J. Zhang. 2004. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* 5:R80.
23. Ploner, C., J. Rainer, H. Niederegger, M. Eduardoff, A. Villunger, S. Geley, and R. Kofler. 2007. The BCL2 rheostat in glucocorticoid-induced apoptosis of acute lymphoblastic leukemia. *submitted*.
24. Megraw, M., P. Sethupathy, B. Corda, and A. G. Hatzigeorgiou. 2007. miRGen: a database for the study of animal microRNA genomic organization and function. *Nucleic Acids Res.* 35:D149-D155.
25. Griffiths-Jones, S., R. J. Grocock, D. S. van, A. Bateman, and A. J. Enright. 2006. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res.* 34:D140-D144.
26. Wu, Z., R. A. Irizarry, R. C. Gentleman, F. Martinez Murillo, and F. Spencer. 2004. A model based background adjustment for oligonucleotide expression arrays. *Internet* http://www.bepress.com/jhubiostat/paper1/.
27. Irizarry, R. A., B. M. Bolstad, F. Collin, L. M. Cope, B. Hobbs, and T. P. Speed. 2003. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res.* 31:e15.
28. Smyth, G. K. 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* 3:Article3.

29. Dudoit, S., J. P. Shaffer, and B. J. Boldrick. 2003. Multiple hypothesis testing in microarray experiments. *Stat. Sci.* 18:71-103.
30. Tang, T. H., N. Polacek, M. Zywicki, H. Huber, K. Brugger, R. Garrett, J. P. Bachellerie, and A. Huttenhofer. 2005. Identification of novel non-coding RNAs as potential antisense regulators in the archaeon Sulfolobus solfataricus. *Mol. Microbiol.* 55:469-481.
31. Thomson, J. M., M. Newman, J. S. Parker, E. M. Morin-Kensicki, T. Wright, and S. M. Hammond. 2006. Extensive post-transcriptional regulation of microRNAs and its implications for cancer. *Genes Dev.* 20:2202-2207.
32. Obernosterer, G., P. J. Leuschner, M. Alenius, and J. Martinez. 2006. Post-transcriptional regulation of microRNA expression. *RNA.* 12:1161-1167.
33. Thulasi, R., D. V. Harbour, and E. B. Thompson. 1993. Suppression of c-*myc* is a critical step in glucocorticoid- induced human leukemic cell lysis. *J. Biol. Chem.* 268:18306-18312.
34. Löffler, M., M. Tonko, B. L. Hartmann, D. Bernhard, S. Geley, A. Helmberg, and R. Kofler. 1999. c-myc does not prevent glucocorticoid-induced apoptosis of human leukemic lymphoblasts. *Oncogene* 18:4626-4631.
35. Cimmino, A., G. A. Calin, M. Fabbri, M. V. Iorio, M. Ferracin, M. Shimizu, S. E. Wojcik, R. I. Aqeilan, S. Zupo, M. Dono, L. Rassenti, H. Alder, S. Volinia, C. G. Liu, T. J. Kipps, M. Negrini, and C. M. Croce. 2005. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc. Natl. Acad. Sci. U. S. A* 102:13944-13949.
36. Pillai, R. S. 2005. MicroRNA function: multiple mechanisms for a tiny RNA? *RNA.* 11:1753-1761.
37. Fazi, F., A. Rosa, A. Fatica, V. Gelmetti, M. L. De Marchis, C. Nervi, and I. Bozzoni. 2005. A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. *Cell* 123:819-831.
38. Landgraf, P., M. Rusu, R. Sheridan, A. Sewer, N. Iovino, A. Aravin, S. Pfeffer, A. Rice, A. O. Kamphorst, M. Landthaler, C. Lin, N. D. Socci, L. Hermida, V. Fulci, S. Chiaretti, R. Foa, J. Schliwka, U. Fuchs, A. Novosel, R. U. Muller, B. Schermer, U. Bissels, J. Inman, Q. Phan, M. Chien, D. B. Weir, R. Choksi, V. G. De, D. Frezzetti, H. I. Trompeter, V. Hornung, G. Teng, G. Hartmann, M. Palkovits, L. R. Di, P. Wernet, G. Macino, C. E. Rogler, J. W. Nagle, J. Ju, F. N. Papavasiliou, T. Benzing, P. Lichter, W. Tam, M. J. Brownstein, A. Bosio, A. Borkhardt, J. J. Russo, C. Sander, M. Zavolan, and T. Tuschl. 2007. A Mammalian microRNA Expression Atlas Based on Small RNA Library Sequencing. *Cell* 129:1401-1414.
39. Kluiver, J., B. J. Kroesen, S. Poppema, and A. van den Berg. 2006. The role of microRNAs in normal hematopoiesis and hematopoietic malignancies. *Leukemia* 20:1931-1936.

Legends to figure

Figure 1: Verification of GC-regulation of miR-15b, 16, and 223 by real time RT-PCR and northern blotting

1A: Ten ng pooled total RNA from CEM-C7H2 cells treated in biological triplicates for 24h with 100nM dexamethasone or 0.1% ethanol as control were reverse-transcribed and PCR-amplified in triplicate using TaqMan® microRNA detection kits for the mature forms of hsa-miR-223, 15b and 16, as indicated. miR-320 served as unregulated control.

1B: Twenty μg pooled total RNA from CEM-C7H2 cells treated in biological triplicates for 12h and 24h with 100nM dexamethasone or 0.1% ethanol as control were size-separated and probed with [^{32}P] labeled probes for the indicated miRNAs. Probes for 5.8 S rRNA served as loading control.

Table 1. GC-regulated miRNAs as detected by microarray-based expression profiling[a]

A. Detection of mature miRNAs - mirVanaTM miRNA Bioarrays

miRNA	M2h	M6h	M24h	A2h	A6h	A24h
miR-15b	0.6/0.6	0.7/0.7	1.3/1.4	10.1/10.1	10.0/10.0	10.9/10.8
miR-16	0.7/0.6	0.9/0.9	1.2/1.2	13.2/13.2	13.0/12.6	13.8/13.8
miR-223	0.7/0.2	0.2/0.2	1.3/1.2	10.0/9.5	9.5/9.4	10.5/10.5
miR-181a	0.9/0.9	1.1/1.1	0.7/0.7	12.9/12.9	12.7/12.7	13.2/13.2
miR-19b	1.4/1.4	1.3/1.5	0.2/0.7	11.3/11.2	10.8/10.6	11.7/11.8

B. Detection of pri- and/or pre-miRNAs - Exon 1.0 microarray

miRNA	probes[b]	M6h	M24h	A6h	A24h	pBH6h[c]	pBH24h[c]
miR-15b	4	0.6	1.7	6.4	6.8	0.99	0.00003
miR-16-2	4	0.1	0.5	5.6	5.7	0.99	0.25293
miR-223	4	0.6	1.3	7.2	7.1	0.99	0.00020
miR-181a-1	4	-0.2	0.3	5.6	5.3	0.99	0.82165
miR-181a-2	4	0.1	-0.2	5.7	5.8	0.99	0.88352
miR-19b-1	4	0.2	-0.3	5.9	5.9	0.99	0.88352
miR-19b-2	4	0.0	0.1	5.0	4.9	0.99	0.88352
mirR-675	1	0.8	1.4	6.7	7.3	0.04	0.00039
miR-19a	4	-0.7	-0.8	6.7	5.7	0.99	0.03695
miR-92-1	4	-0.1	-1.2	7.7	7.5	0.99	0.00628

C. Detection of pri-miRNAs - U133 Plus 2.0 microarray

miRNA	gene	probe set[d]	M6h	M24h	A6h	A24h
miR-15b~16-2	SMC4	201664_at	0.5	2.1	12.1	12.9
miR-19a~92-1[e]	C13orf25	232291_at	-0.3	-2.0	7.7	6.9
miR-223	LOC389865	229934_at	2.2	3.0	8.3	8.7

Footnotes to Table 1:

[a]M and A: M-values (log2 fold change values with +/- indicating up- or downregulation, respectively) and A-values (arithmetic mean of the log2 expression levels of the compared samples) obtained after 6h (M6h, A6h) or 24h (M24h, A24h) of GC treatment. Shown are values for both replicated spots (Ambion arrays, Table 1A), mean values for triplicates (Exon array,

Table 1B) or individual values (U133 Plus 2.0, Table 1C). M values >1.0 and < -1.0 (Tables 1A and C), and mean M values >0.7 and < -0.7 and pBH values <0.05 (Table 1B) are high-lighted.

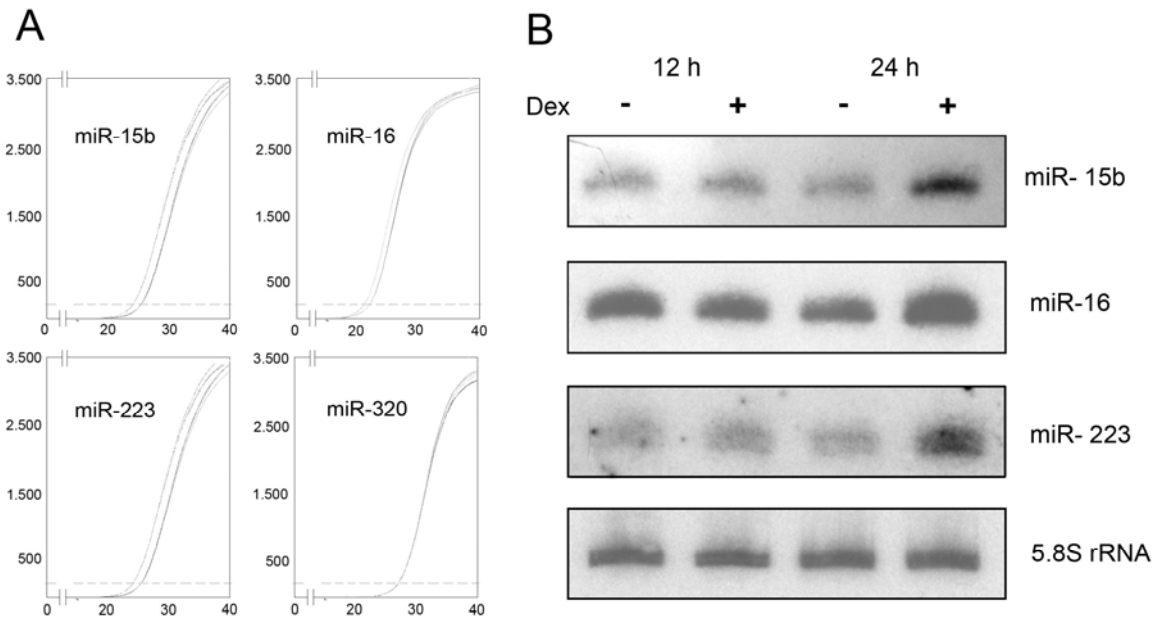
[b]number of individual probes recognizing the respective pre-miRNA sequence

[c]pBH, Benjamini Hochberg-adjusted p-values

[d]The most 3' located probe set on the U133 plus 2.0 array (resulting in the best signal) is depicted

[e]C13orf25 encodes miR-17-5p~18a~19a~20a~19b-1~92-1.

Figure 1: Verification of GC-regulation of miR-15b, 16, and 223 by real time RT-PCR and northern blotting



The BCL2 rheostat in glucocorticoid-induced apoptosis of acute lymphoblastic leukemia

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ABSTRACT

Glucocorticoid (GC)–induced apoptosis is an essential component in the treatment of acute lymphoblastic leukemia (ALL) and related malignancies. Pro- and anti-apoptotic members of the large BCL2 family control many forms of apoptotic cell death, but the extent to which this survival "rheostat" is involved in the beneficial effects of GC therapy is not understood. We performed a systematic analysis of expression, GC regulation and function of BCL2 molecules in primary ALL lymphoblasts and corresponding *in vitro* models. Affymetrix-based expression profiling revealed that the response included regulations of pro- and, surprisingly, anti-apoptotic BCL2 family members, varied among patients, but was dominated by induction of the BH3-only molecules, BMF and BCL2L11/Bim. Conditional lentiviral gene over-expression and knock-down by RNA interference in the CCRF-CEM model revealed that induction of Bim, and to a lesser extent that of BMF, was required and sufficient for apoptosis. Although anti-apoptotic BCL2 members were not regulated consistently by GC in the various systems, their over-expression delayed, whereas their knock-down accelerated, GC-induced cell death. Thus, the combined clinical and experimental data suggest that GCs induce both pro- and anti-apoptotic BCL2 family member-dependent pathways with the outcome depending on cellular context and additional signals feeding into the BCL2 rheostat.

INTRODUCTION

Glucocorticoids (GCs) induce apoptosis in certain lymphoid cells and play an important role in the treatment of childhood acute lymphoblastic leukemia (ALL) and other lymphoid malignancies[1,2]. This effect is mediated by the GC receptor (GR), a ligand-activated transcription factor of the nuclear receptor super-family that resides in the cytoplasm and, upon ligand binding, translocates into the nucleus, where it modulates gene expression via binding to specific DNA response elements or by protein-protein interactions with other transcription factors[3]. A large number of genes has been identified that are regulated by GC in experimental systems of GC-induced apoptosis[4] and related clinical samples[5,6], but the genes responsible for cell death induction remain controversial (for recent reviews see)[4,7-12].

GC might induce cell death by directly regulating genes controlling cell survival and apoptosis, or *via* (de)regulating genes or gene networks leading to cellular distress that, in turn, constitutes an apoptotic stimulus. In both scenarios, members of the large family of pro- and anti-apoptotic BCL2 proteins[13,14], referred to as the "BCL2 rheostat"[15], might be involved either as direct GR targets or as sensors for potentially harmful GC effects[16]. In addition, the status of the BCL2 rheostat, regardless of whether altered during GC exposure or not, might define sensitivity to, and kinetics of, GC-induced cell death. The latter issue was addressed in great detail in mice showing that GC-induced thymocyte apoptosis was impaired by transgenic expression of anti-apoptotic or knock-out of some pro-apoptotic BCL2 family members (reviewed in)[7,13,14,17]. In human ALL, only a few of these genes have been functionally tested in this respect. For instance, over-expression of BCL2[18,19] and knock-down of the BCL2 homology domain 3 (BH3)–only molecule BCL2L11/Bim[20,21] interfered with, while over-expression of pro-apoptotic BAX[22] and knock-down of anti-apoptotic MCL1[23] sensitized for, GC-induced apoptosis in ALL cell lines

(following a recommendation by the HUGO Gene Nomenclature Committee, all official gene symbols are represented by uppercase letters to distinguish them from their alternatives, e.g., BCL2L11 = Bim). To what extent, if any, expression of BCL2 family proteins predicts GC sensitivity in patients with ALL is controversial (discussed in)[4,8,11], although MCL1 has recently been suggested as major GC resistance gene that specifically protects ALL cells from GC-, but not chemotherapy-induced, apoptosis[23]. Taken together, current evidence, strong in mice but less convincing in human systems, suggests that the status of the BCL2 rheostat influences GC-sensitivity.

Concerning the question of whether components of the BCL2 rheostat might be regulated by GC, several BCL2 family members responded to GC in numerous systems of GC-induced apoptosis, most notably Bim, which was induced in mouse thymocytes[5,24], several leukemia cell lines[4,7,24], primary chronic lymphocytic leukemia cells[25] and some patients with ALL[5]. Other reported regulations include BMF and Puma mRNA induction in mouse thymocytes[5,26] or BCL2 and Bcl-XL protein repression in children with ALL[27]. However, in a recent study with primary ALL cells from children treated with GC *ex vivo*, neither Bim nor any other BCL2 family member was significantly regulated[6]. The most critical question, i.e., to what extent the BCL2 rheostat responds to GC treatment in patients *in vivo*, has not been thoroughly addressed.

In this study, we performed a systematic analysis of mRNA expression and GC regulation of all BCL2 family members during the early phase of systemic GC mono-therapy in children with ALL and complemented it with extended functional analyses in CCRF-CEM cells, probably the most widely-used model for GC-induced apoptosis in childhood ALL. Specifically, we asked (1) which members of the BCL2 family were expressed in ALL cells and regulated in the clinical response to GC, (2) what the functional relevance of these regulations for cell death induction

was, and (3) to what extent sensitivity and kinetics of GC-induced leukemia apoptosis depended on the status of the BCL2 rheostat prior to treatment initiation *in vitro*.

MATERIALS AND METHODS

Cell lines, tissue culture and apoptosis determination

PreB697[28], CCRF-CEM-C7H2[29], CEM-C7H2-2C8 (expressing the tetracycline-responsive reverse transactivator rtTA)[30], and their below described derivatives were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 100U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine at 37ºC, 5% carbon-dioxide and saturated humidity. Phoenix retroviral packaging cells (kindly provided by G.P. Nolan)[31] and 293T packaging cells for lentivirus production (obtained from ATCC) were grown in DMEM containing 10% FCS, 100U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine. All cell lines were tested for, and found to be free of, mycoplasma infection. The authenticity of all cell lines was verified by DNA fingerprinting, as detailed previously[32]. Dexamethasone (10mM in 100% ethanol), puromycin (10mg/ml in water) and doxycycline (1mg/ml in phosphate buffered saline) were obtained from Sigma (Vienna, Austria).

Apoptosis was determined by propidium iodide (PI) staining of nuclei[33] as detailed previously[34].

Expression profiling, data analysis and statistics

Forty eight whole genome (U133 plus 2.0) expression profiles from peripheral lymphoblasts of 3 T-ALL and 10 precursor B-cell ALL children and an adult with precursor B-ALL prior to and 6-8h and 24h after GC exposure, and similarly treated CEM-C7H2 and PreB697 cells[5] were re-analyzed using GCOS for image analysis, GCRMA (robust multi-array average with background

adjustment using sequence information)[35] for data pre-processing and normalization, and Affymetrix NetAffx annotation version 21 for annotation. Pre-processing and subsequent analysis were performed in R (http://www.R-project.org) version 2.4.1 using Bioconductor[36] version 1.9. All probe sets annotated to the 21 Bcl-2 family members (Table S1) by Affymetrix were re-annotated with respect to their localization along the known mRNAs assigned to these genes in Genbank and only probe sets residing within ~600bps from the 3´end of the respective transcripts were included in the final analyses (see supplement).

For Exon array-based expression profiling, three biological replicates of CEM-C7H2 and PreB697 cells treated with 100nM dexamethasone or 0.1% ethanol as carrier control for 6h and 24h were used. Target preparation is detailed in the supplement. In brief, total RNA was depleted of ribosomal RNA and transcribed into cDNA using T7-promoter-tagged hexamer primers. Antisense RNA was produced by T_7-polymerase and transcribed into cDNA using random priming in the presence of dUTP which was used to enzymatically fragment the cDNA. The resulting targets were labeled by addition of biotin-linked deoxynucleotides with terminal deoxynucleotidyl transferase, hybridized to Human Exon 1.0 ST arrays, washed and stained in an Affymetrix 450S fluidics station, and read by an Affymetrix 3000 scanner. Data from the resulting 24 arrays were RMA- (robust multi-array average)[37] pre-processed and type 1 core probe sets summarized to one expression value per individual RefSeq sequence (see Table S1 for probe sets). Raw p-values were calculated with the moderated t-test[38] and adjusted for multiple hypothesis testing according to Benjamini-Hochberg[39]. Data validation and quality controls are presented in the supplement.

Vectors and stable cell lines

For functional analyses of Bim, BMF, BCL2, Bcl-X and MCL1, we generated Gateway-compatible "destination" vectors derived from the lentiviral plasmid pHR-SIN-CSGW-ΔNot[40]. For conditional cDNA over-expression, pHR-tetCMV-Dest-ires-mGFP5 was generated by ligating the tetCMV-DEST-ires-mGFP5 cassette into the NotI-EcoRI site of pHR-SIN-CSGW-ΔNot. This cassette contains the tetracycline- responsive tetCMV promoter from pUHD-10-3[41], a AttR-site flanked ccdB-CM cassette ("DEST cassette", Invitrogen, Carlsbad, CA), an encephalo-myocarditis-virus IRES element[42], and mGFP5[43]. cDNAs were recombined into pENTR207 plasmids and subsequently into pHR-tetCMV-Dest-ires-mGFP5. For RNA interference-mediated gene knock-down, shRNA oligonucleotides directed against either Bim, BMF, MCL1, BCL2, or Bcl-X (Table S4) were cloned into pENTR-THT containing a tetracycline-sensitive RNAseP H1 promoter for regulated the shRNA expression. The THT-shRNA cassette was recombined into the lentiviral destination vector pHR-Dest-SFFV-eGFP, which was generated by insertion of a DEST cassette into the blunt-ended EcoRI site of pHR-SIN-CSGW-ΔNot. Negative controls included numerous constructs containing shRNAs against genes such as *PFKFB2* or *SOCS1* that failed to show any effect on kinetics or sensitivity of GC-induced apoptosis.

The above constructs were transfected into 293T packaging cells and viral particle-containing supernatants were used to infect rtTA expressing C7H2-2C8[30] for gene over-expression or tetR-KRAB expressing C7H2-2B10 cells for gene knock-down. Stable cell clones were generated by limiting dilution cloning. For Bcl-XL over-expression, cDNA encoding Bcl-XL was cloned into the XbaI-BglII site of the retroviral vector pLIB-MCS2-ires-Puro[44].

Immunoblotting and immunoprecipitation

For immunoblotting, rabbit polyclonal antibodies recognizing Bim, Bcl-XL, BID, BAD, Puma, MCL1, cyclin D3, FKBP51, GR, or monoclonal antibodies recognizing Noxa, BCL2, BMF, and α-tubulin were used. For immunoprecipitation, cell lysates were incubated with either anti-MCL1 rabbit antiserum or normal rabbit serum, and the complexes were isolated via Protein A beads and subjected to immunoblotting with antibodies against MCL1 and Bim.

RESULTS

The BCL2 rheostat in the initial phase of GC treatment

To assess expression and possible regulation of the BCL2 rheostat in response to initial GC therapy in patients with ALL, we utilized our recently established expression profiles (Affymetrix, U133 plus 2.0) of lymphoblasts from 13 ALL children prior to, and 6-8h and 24h after, initiation of GC treatment[5]. First, all arrays were re-normalized using GCRMA[35], which has been shown to be superior compared to the previously used RMA (robust multiarray analysis) normalization procedure[45]. Second, we checked all probe sets (collection of eleven 25mer oligonucleotides on the array that recognize a given transcript) assigned to the 21 genes of the BCL2-family[13,14] for correct annotation and position along the transcripts and found proper probe sets (i.e., probe sets that mapped within ~600bps of the 3´ end of the respective transcript) for all 21 genes and almost all of their 42 RefSeq transcripts. The exceptions were 1 variant each of BCL2L14/Bcl-G and BCL2L11/Bim, and 3 variants of the C1orf178/Bfk gene (supplemental Table S1). Prior to treatment, MCL1 and BNIP3L were strongly expressed in all 13 childhood ALL samples, whereas BCL2L10/Boo/Diva, BOK, and Bcl-G were not detectable in any of them

(Figure 1A). The mRNA expression pattern seen in the children was largely maintained in an adult ALL sample and, to a lesser degree, in the CCRF-CEM and PreB697 ALL *in vitro* models. The mean regulations (M-values, log2 fold changes) of the rheostat components in the childhood ALL samples along with their significance (p_{BH}, p-values adjusted for multiple hypothesis testing according to Benjamini and Hochberg)[39] are depicted in the "volcano plots" in Figure 1B, which visualize regulations on the X-axis and significance of these regulations on the Y-axis. At the early time point (6-8h after treatment initiation), the BH3-only molecule BMF was the only significantly regulated BCL2 family member (p_{BH}=0.004, mean M=0.8), but 4 additional genes came close to the significance cut-off p_{BH}=0.05, including the pro-survival gene BCL2A1/A1 (p_{BH}=0.057) and the pro-apoptotic HRK (p_{BH}=0.057) and PMAIP1/Noxa (p_{BH}=0.055) genes. Interestingly, A1 (mean M=0.7) was induced and HRK (mean M= -0.3) and Noxa (mean M= -1.0) were repressed, presumably reflecting GC-mediated pro-survival signals. The 4th transcript, encoding the pro-apoptotic BAK1 molecule (p_{BH}=0.055), was induced, but the extent of regulation was very low (mean M=0.2). After 24h, the significance and mean M values of A1, HRK and BAK1 decreased and another pro-apoptotic transcript was repressed (BAX, mean M= -0.5, p_{BH}=0.038). BMF regulation, in contrast, became more pronounced (mean M=1.2, p_{BH}=0.003) and another pro-apoptotic transcript, Bim, reached significance levels (p_{BH}=0.038, mean M=1.1).

Since statistical procedures like the one above may obscure events in subgroups of patients, we also analyzed the response of individual children along with that of an adult with preB-ALL (supplemental Table S7). Six BCL2 family members were >2-fold regulated in 4 or more patients, namely BCL2, A1, HRK, Noxa, Bim and BMF, however, BMF and Bim showed the most consistent regulations, i.e., 11/14 patients induced BMF and/or Bim (M≥1) and 2 of the

remaining 3 showed borderline regulation (M=0.8 and 0.9, after 24h). Thus, the clinical response of the BCL2 rheostat to GC treatment was dominated by induction of the pro-apoptotic BH3-only members BMF and Bim, but was otherwise heterogeneous.

Impact of GC treatment on the BCL2 rheostat in ALL *in vitro* models

To get a deeper insight into the response of the BCL2 rheostat to GC and to determine how closely our *in vitro* models resembled the clinical situation, we subjected GC-treated CEM-C7H2 and PreB697 cells to whole genome expression profiling. Although we had previously analyzed both cell lines on the U133 plus 2.0 array[5](see Figure 1A), we reanalyzed them now using the recently introduced Affymetrix Exon array technology to obtain additional information not provided by conventional expression profiling (see discussion). Moreover, all assays were now performed in triplicate to improve data quality. CEM-C7H2 cells underwent almost complete apoptosis after 48h-72h of GC exposure (100nM dexamethasone), whereas PreB697 apoptosis was slower and less complete (Figure 2). For Exon array analysis, we selected the same time point as for the clinical samples (6h and 24h GC exposure). The resulting 24 expression profiles were searched for probe sets corresponding to the 42 BCL2 family member transcripts contained in the RefSeq data base (supplemental Table S1). Unlike the patients, PreB697 cells did not significantly regulate any BCL2 member after 6h GC exposure (Figure 1C). After 24h, Bim was significantly induced (M=0.8, p_{BH}=0.0001), Noxa, BID, BAX and BCL2 were down-regulated, but with lower M- and/or less significant p_{BH}-values; BMF, however, was not regulated. CEM-C7H2 cells, in contrast, showed strong and significant induction of Bim as early as 6h (M=1.2; p_{BH}=0.01), and after 24h, regulation of several others, including BMF (complete data are shown in supplemental Table S8).

Basal expression and GC-regulation of the pro-apoptotic BH3-only molecules BAD, Bim, BMF, BBC3/Puma, Noxa and BID, and the anti-apoptotic proteins BCL2L1/Bcl-X, BCL2 and MCL1 were confirmed in CCRF-CEM cells on the protein level (Figure 3). Eight of 9 investigated BCL2 proteins were detectably expressed in CCRF-CEM cells, which correlated well with mRNA expression levels on the arrays (supplemental Table S3). Similarly, with the exception of Puma, the protein regulations were paralleled by corresponding up- or down-regulations on the RNA level, albeit to varying degrees. Thus, Bim mRNA was ~2 to 4-fold induced with high statistical significance (p_{BH} values: 0.010 at 6h, $4.1x10^{-9}$ at 24h). MCL1, Noxa and BMF were significantly regulated as well (p_{BH}-values of 0.0043, 0.0091 and 0.011, respectively), but showed considerably lower M values (0.5, -0.6, 0.4, respectively), and the slight repression of BCL2 and Bcl-X mRNA (M= -0.2 and -0.3, respectively) was not statistically significant. Taken together, both tested *in vitro* models resembled the response in the patients, with CEM-C7H2 mimicking the clinical situation more closely since it responded as early as 6h and induced both Bim and BMF, and was therefore selected for functional analyses.

Functional significance of Bim and BMF induction

To determine whether Bim and/or BMF induction is required for GC-induced cell death, we conditionally knocked down the expression of each of the 2 molecules in CCRF-CEM cells. For this purpose, we first generated a CCRF-CEM derivative expressing the tetracycline-responsive transrepressor tetR-KRAB (CEM-C7H2-2B10), transduced it with lentiviral constructs expressing shRNAs directed against Bim or BMF in a tetracycline-dependent manner and generated stable derivatives by limiting dilution cloning. For each knock-down, 2 clones were analyzed (termed 2B10/Bim-shRNA#1, 2B10/Bim-shRNA#2, 2B10/BMF-shRNA#1, and 2B10/BMF-shRNA#39). These clones had maintained a normal GC response, as suggested by

intact GR auto-induction, induction of FKBP51 and repression of cyclin D3 (supplemental Figure S2), three known GC response genes in lymphoid cells[46-49]. After induction of shRNA expression with doxycycline, both Bim-shRNA clones showed reduced basal and GC-induced levels of the target protein, with 2B10/Bim-shRNA#1 showing somewhat better knock-down than 2B10/Bim-shRNA#2 (Figure 4A, left panel). Bim knock-down was associated with a corresponding reduction in GC-induced apoptosis at 48h (Figure 4B, left panel). The protective effect vanished after another 24h, which may be explained by the inability of the shRNA to maintain Bim repression in the continuous presence of GC (data not shown) and/or by other pro-apoptotic regulatory events, such as BMF induction (see below). Knock-down of BMF almost completely prevented its induction by GC (Figure 4A, right panel), however, the protection from GC-induced cell death was less complete than that observed with Bim knock-down (Figure 4B, right panel). Taken together, the data suggested that induction of both Bim and BMF contribute to GC-induced apoptosis but that, in this model, Bim responds earlier and contributes more significantly to death induction than BMF.

To assess whether induction of Bim or BMF alone is sufficient to induce cell death, we generated stable derivatives of CEM-C7H2-2C8 cells (which constitutively express the tetracycline-regulated transactivator protein rtTA)[30] by lentiviral transduction with constructs enabling tetracycline-induced expression of transgenic BimEL (NM_138621) and BMF-1 (NM_001003940), the 2 major GC-regulated isoforms in this system (Figure 3). In these cell lines, the level of transgene expression could be controlled at will by varying the amount of doxycycline (Figure 5A). Using this system, both BimEL and BMF-1 over-expression led to significant cell death in a dose-dependent manner (Figure 5B), proving the ability of these molecules to induce apoptosis in the model cell line and, by extrapolation, probably in ALL cells from patients as well.

Bim binds to and stabilizes MCL1

The marked induction of the MCL1 protein that followed the kinetics of the Bim protein induction, but not that of its mRNA (Figures 1C and 3C), raised the questions of whether MCL1 induction was related to that of Bim, and why MCL1 induction did not prevent apoptosis. In response to the former, GC induction of the MCL1 protein was reduced when the parallel induction of Bim was prevented by shRNA-mediated knock-down (Figure 6A). Moreover, tetracycline-induced Bim expression resulted in increased MCL1 protein in the absence of GC (Figure 6B). Taken together, these data indicate that the increase of MCL1 protein observed after GC exposure was, at least in part, a consequence of GC-mediated induction of Bim, presumably via MCL1 stabilization, as suggested by monitoring its expression in cycloheximide-treated cells in the absence or presence of GC (Figure 6C). The latter conclusion was further supported by the recent observation that transfected human BimS caused endogenous Mcl-1 protein stabilization in mouse embryonic fibroblasts[50]. The failure of increasing MCL1 protein to block apoptosis can be explained by the observation that significantly more Bim co-immunoprecipitated with MCL1 after GC treatment (Figure 6D). Thus, unlike Noxa, which binds to MCL1 and facilitates its degradation[51], Bim appears to bind to and inactivate MCL1 without degradation.

Effect of the BCL2 rheostat on GC sensitivity

To determine how the status of the BCL2 rheostat prior to treatment affects sensitivity and kinetics of GC-induced apoptosis, we genetically manipulated the expression levels of MCL1, Bcl-XL and BCL2, the 3 anti-apoptotic BCL2 family members expressed in this ALL model (Figures 1 and 3). First, we generated clonal CCRF-CEM derivatives with conditional knock-down of either of the 3 molecules. Doxycycline exposure for 72h to 96h led to a significant reduction of the expression of MCL1, Bcl-XL and BCL2, respectively (Figure 7A). This

reduction alone had no detectable effect on cell cycle progression and viability during the first 72h, but in all three instances, the cells became more sensitive to GC-induced apoptosis and the kinetic of the response was accelerated (Figure 7B). The effect was most pronounced with MCL1 knock-down where cells died 24h earlier and responded to as little as 10nM dexamethasone. Bcl-XL knock-down showed an intermediary response and that of BCL2 the weakest.

In the second approach, we investigated whether increased expression of these molecules protected against GC-induced apoptosis. We had previously reported that doxycycline-controlled BCL2 expression delayed GC-induced cell death in CEM-C7H2 cells by about 24h[18]. Figure 7 (C and D) shows a similar effect for both Bcl-XL and MCL1 with the degree of protection roughly correlating with the amount of transgene expression. Thus, among the MCL1 over-expressing cell lines, 2C8/MCL1#13 cells showed the least transgene induction upon doxycycline exposure and the weakest protection. 2C8/MCL1#11 and #20 expressed more MCL1 and were better protected. The 3 Bcl-XL cell lines expressed their transgene at very high levels, which entailed an almost 48h delay in GC-induced apoptosis. Taken together, these data indicated that expression levels of all 3 anti-apoptotic BCL2 family members prior to GC treatment influence sensitivity and kinetics of the GC-induced cell death response.

DISCUSSION

The present study delineated, for the first time, expression and regulation of the entire BCL2 rheostat in the early phase of GC therapy in ALL patients and compared it with the corresponding response in two well-defined ALL *in vitro* models. The clinical response differed between individual patients and encompassed both pro-and anti-apoptotic signaling. Nevertheless, the BH3-only genes *BMF* and/or *Bim* were induced in the vast majority of ALL children as well as

an adult with ALL. Interestingly, as shown in the supplemental Table S7, a poorly defined transcript from the *BCL2L11* locus, termed *Bam*[52], was induced (M≥1) in 10/14 patients. This transcript starts in the intron preceding the BH3 containing exon 8 of the *Bim* gene and codes for a predicted 73 amino acid protein that shares its BH3 domain with Bim but has a unique C-terminus. If *Bam* is included and the regulation cut-off is lowered from M=1 to M=0.8, all patients induced either one of the 3 BH3-only containing transcripts within the first 24h after initiation of systemic GC mono-therapy.

Whether BMF and Bim induction is necessary and/or sufficient for cell death was addressed in the CCRF-CEM childhood ALL model. Despite the known limitations, an *in vitro* model had to be used because functional analyses like the ones performed in this study cannot currently be performed with primary cells because of technical difficulties and the fact that ALL blasts undergo rapid spontaneous apoptosis *in vitro*. That transgenic Bim caused apoptosis was not surprising since Bim is considered one of the most potent pro-apoptotic molecules in both current BCL2 rheostat models. In the "direct activator/de-repressor model"[53,54] it acts as direct activator of BAX, and in the "displacement model"[17,55,56] it is a potent neutralizer of all 5 BCL2-like pro-survival proteins. That BMF on its own, and at levels comparable to those seen after GC induction (Figures 3 and 4), sufficed for apoptosis induction was less predictable. In the first model, sole induction of BMF as a "de-repressor" should not entail cell death, however, since a "direct activator" (in our case Bim, Figure 3) is already present in the system, a "de-repressor" might suffice for cell death induction[57]. In the "displacement model" BMF is considered a weak death agonist that only neutralizes BCL2, Bcl-X or Bcl-W, but not A1 or MCL1[58], the latter being well expressed in CCRF-CEM cells (Figures 1 and 3). However, since Bim and Noxa are expressed as well (Figure 3) and may neutralize MCL1, transgenic BMF might kill by freeing

pro-apoptotic BAK out of its complex with Bcl-XL as recently suggested[51]. In any rate, if the data in the CCRF-CEM model can be extended to the clinical situation, the induction of BMF and/or Bim explains GC-induced cell death in the majority of patients on a mechanistic level. In this context it is worth mentioning that *Puma* mRNA, that is induced by GC in mouse thymocytes *in vivo* and *ex vivo*[5,26] and whose knock-out impairs GC-induced thymocyte apoptosis[59,60], was not regulated in patients (Table S7) or cell lines (Table S8). Thus, the human *Puma* gene is not a transcriptional target of the GR, and if Puma contributes to GC-induced apoptosis in human ALL, it does so in a transcription-independent manner.

A question of considerable clinical relevance is whether expression of BCL2 family proteins predicts GC responsiveness in ALL patients. The experiments in Figure 7 considered together with similar data in the literature[18-22] indicate that the status of the BCL2 rheostat prior to treatment affects the kinetics of, and sensitivity to, GC-induced apoptosis in experimental systems. In contrast, the literature concerning this issue in patients has been controversial[4,8,11], although a recent report combining bioinformatic analyses of expression profiles from ALL children (classified as GC-sensitive/resistant by *ex vivo* testing) with functional analyses in experimental systems identified MCL1 as the key anti-apoptotic BCL2 family protein responsible for GC resistance[23]. However, direct proof that MCL1 expression, but not that of other pro-survival proteins, predicts and causes *in vivo* resistance to GC therapy is still lacking. Interestingly in this respect, a recent genome-wide gene expression comparison between precursor B-cell blasts at diagnosis and after 8d systemic GC mono-therapy showed differential expression of a single BCL2 family member, i.e, BCL2 that was surprisingly reduced in day 8 blasts which are considered to be GC resistant[61].

In addition to pro-apoptotic signals (induction of Bim and BMF, repression of BCL2 and Bcl-XL), numerous patients and both cell lines showed anti-apoptotic regulations as well, most

impressively a marked reduction of the BH3-only molecule Noxa, a specific antagonist of MCL1 implicated in growth factor withdrawal and nutrient shortage-induced cell death in the lymphoid lineage[62]. This is reminiscent of the well-documented potential of GC to elicit cell death in some, but be protective in other, cell types[4,63]. In the CCRF-CEM model, and perhaps in patients as well, anti-apoptotic and pro-apoptotic signaling may even occur within the same cell. The CCRF-CEM model may exemplify just one possible scenario: Noxa reduction may free MCL1 that in turn serves as a buffer for increasing Bim levels thereby preventing cell death within the first 24h. Thereafter, additional pro-apoptotic regulations (e.g., further increase of Bim, induction of BMF and/or Puma, repression of BCL2 and/or Bcl-XL) might tip the balance. Thus, the cellular context and additional signals feeding into the BCL2 rheostat may ultimately determine which BCL2 family members participate in this antagonistic interplay, to what extent and in which direction they are being regulated, and whether survival or cell death ensues.

Of considerable interest for understanding GC-induced cell death signaling is whether the regulated BCL2 genes are direct GC targets or secondary to some other upstream GC-regulated signal(s). Unfortunately, this point could not be resolved by a bioinformatic search for GC response elements (GREs) in the promoters of these genes because corresponding programs (e.g., Genomatix, Match) failed to reliably discriminate between positive control genes (i.e., genes containing GREs supported by experimental evidence) and negative controls (i.e., genes that were well expressed but showed no regulation in any of our comparisons). We next performed a PubMed literature mining in R using Bioconductors annotate package and also using the commercial tool Bibliosphere from Genomatix, however, functional GREs have thus far been described in only two BCL2 family genes, i.e., mouse Bcl-X[64-66] and rat Bnip3[67]. Interestingly, no reports concerning the most significantly regulated BCL2 family members in patients and cell

lines (BMF, Bim, and Noxa) were detected. Moreover, no BCL2 family members were found among 318 GR-bound promoter regions in livers from mice treated intraperitoneally with dexamethasone[68]. Thus, the question of whether regulation of the BCL2 rheostat (that precedes apoptosis induction at least in cell lines) is the most upstream event in GC-induced apoptosis remains unresolved.

This study reports, for the first time, expression profiles from ALL cell lines during GC exposure using the recently introduced Exon array technology to identify GC response genes. The corresponding data are now available for interested investigators through the internet (www.ncbi.nlm.nih.gov.geo, accession number GSE7446). Data from such arrays can be analyzed in a number of ways. We present data based on summarizing "core" probe sets of verified full length transcripts (RefSeq) of the BCL2 family (Figure 1C and supplemental Table S8). These data, as well as analysis of all RefSeq transcripts on the array (unpublished results) revealed a good correlation with the data obtained by the conventional 3' end arrays (U133 plus 2.0, supplemental Table S3). The advantage of Exon arrays in this application is that they interrogate the entire transcript whereas conventional arrays reliably detect only sequences at the 3' end of the transcript. A disadvantage was the reduced dynamic range in the former compared to the latter (supplemental Table S3). A more detailed bioinformatic work-up of the data using different summarization procedures to identify splice variants and predicted transcripts is currently under way in our lab. This exploratory use of the Exon array exploits its real potential, however, the bioinformatic requirements for this task are demanding.

In conclusion, our study suggests a model in which GCs affect, directly or indirectly, expression of the BCL2 rheostat, in particular that of the BH3-only molecules BMF, Bim and Noxa thereby controlling the activity of anti-apoptotic BCL2 molecules, such as MCL1, and the

killer proteins BAX and/or BAK. Dependent on cellular context (including levels and responsiveness of the GR[69], its phosphorylation status[70-72], expression of *BCL2* genes prior to treatment and additional signals feeding into the rheostat), this effect may lead to different sensitivity to, and kinetics of, GC-induced cell death. With the advent of effective antagonists of anti-apoptotic BCL2 proteins, the emerging understanding of the BCL2 rheostat prior to and during therapy of ALL cells may become relevant for innovative therapy[57].

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Supplemental material

A more detailed description of expression profiling and vector constructions is provided in the supplement (at the end of this manuscript) along with 8 Tables and 2 Figures.

Data access via the internet:

All raw and normalized data from the 48 U133 plus 2.0 and the 24 Human Exon 1.0 ST microarrays can be accessed via Gene Expression Omnibus (GEO):

http://www.ncbi.nlm.nih.gov/geo, accession number GSE7446.

Conflict of interest: The authors have no conflict of interest to declare.

Authors contribution:

C. Ploner designed and performed the functional analyses and contributed to the writing of the manuscript. J. Rainer and M. Eduardoff performed the bioinformatic data analyses, H. Niederegger contributed to expression profiling and data presentation, S. Geley and A. Villunger assisted in writing and data interpretation, and R. Kofler designed and co-ordinated the entire study and wrote the manuscript. The final version was seen and approved by all authors.

REFERENCES

1. Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. N.Engl.J.Med. 2004;350:1535-1548.

2. Schrappe M, Reiter A, Ludwig WD et al. Improved outcome in childhood acute lymphoblastic leukemia despite reduced use of anthracyclines and cranial radiotherapy: results of trial ALL-BFM 90. German-Austrian-Swiss ALL-BFM Study Group. Blood 2000;95:3310-3322.

3. Laudet V, Gronemeyer H. The nuclear receptor facts book. London: Academic Press; 2002.

4. Schmidt S, Rainer J, Ploner C et al. Glucocorticoid-induced apoptosis and glucocorticoid resistance: Molecular mechanisms and clinical relevance. Cell Death Differ. 2004;11 Suppl 1:S45-S55.

5. Schmidt S, Rainer J, Riml S et al. Identification of glucocorticoid response genes in children with acute lymphoblastic leukemia. Blood 2006;107:2061-2069.

6. Tissing WJ, den Boer ML, Meijerink JP et al. Genome-wide identification of prednisolone-responsive genes in acute lymphoblastic leukemia cells. Blood 2007;109:3929-3935.

7. Distelhorst CW. Recent insights into the mechanism of glucocorticosteroid-induced apoptosis. Cell Death Differ. 2002;9:6-19.

8. Tissing WJ, Meijerink JP, den Boer ML, Pieters R. Molecular determinants of glucocorticoid sensitivity and resistance in acute lymphoblastic leukemia. Leukemia 2003;17:17-25.

9. Frankfurt O, Rosen ST. Mechanisms of glucocorticoid-induced apoptosis in hematologic malignancies: updates. Curr.Opin.Oncol. 2004;16:553-563.

10. Schaaf MJ, Cidlowski JA. Molecular mechanisms of glucocorticoid action and resistance. J.Steroid Biochem.Mol.Biol. 2002;83:37-48.

11. Haarman EG, Kaspers GJ, Veerman AJ. Glucocorticoid resistance in childhood leukaemia: mechanisms and modulation. Br.J.Haematol. 2003;120:919-929.

12. Planey SL, Litwack G. Glucocorticoid-induced apoptosis in lymphocytes. Biochem.Biophys.Res.Commun. 2000;279:307-312.

13. Strasser A. The role of BH3-only proteins in the immune system. Nat.Rev.Immunol. 2005;5:189-200.

14. Ranger AM, Malynn BA, Korsmeyer SJ. Mouse models of cell death. Nat.Genet. 2001;28:113-118.

15. Oltvai ZN, Korsmeyer SJ. Checkpoints of dueling dimers foil death wishes. Cell 1994;79:189-192.

16. Almawi WY, Melemedjian OK, Jaoude MM. On the link between Bcl-2 family proteins and glucocorticoid-induced apoptosis. J.Leukoc.Biol. 2004;76:7-14.

17. Labi V, Erlacher M, Kiessling S, Villunger A. BH3-only proteins in cell death initiation, malignant disease and anticancer therapy. Cell Death.Differ. 2006;13:1325-1338.

18. Hartmann BL, Geley S, Löffler M et al. Bcl-2 interferes with the execution phase, but not upstream events, in glucocorticoid-induced leukemia apoptosis. Oncogene 1999;18:713-719.

19. Brunet CL, Gunby RH, Benson RSP et al. Commitment to cell death measured by loss of clonogenicity is separable from the appearance of apoptotic markers. Cell Death Differ. 1998;5:107-115.

20. Abrams MT, Robertson NM, Yoon K, Wickstrom E. Inhibition of glucocorticoid-induced apoptosis by targeting the major splice variants of BIM with siRNA and shRNA. J.Biol.Chem. 2004;279:55809-55817.

21. Lu J, Quearry B, Harada H. p38-MAP kinase activation followed by BIM induction is essential for glucocorticoid-induced apoptosis in lymphoblastic leukemia cells. FEBS Lett. 2006;580:3539-3544.

22. Salomons GS, Brady HJM, Verwijs-Janssen M et al. The Baxα:Bcl-2 ratio modulates the response to dexamethasone in leukaemic cells and is highly variable in childhood acute leukaemia. Int.J.Cancer 1997;71:959-965.

23. Wei G, Twomey D, Lamb J et al. Gene expression-based chemical genomics identifies rapamycin as a modulator of MCL1 and glucocorticoid resistance. Cancer Cell 2006;10:331-342.

24. Wang Z, Malone MH, He H, McColl KS, Distelhorst CW. Microarray analysis uncovers the induction of the pro-apoptotic BH3-only protein Bim in multiple models of glucocorticoid induced apoptosis. J.Biol.Chem. 2003;278:23861-23867.

25. Iglesias-Serret D, de FM, Santidrian AF et al. Regulation of the proapoptotic BH3-only protein BIM by glucocorticoids, survival signals and proteasome in chronic lymphocytic leukemia cells. Leukemia 2007;21:281-287.

26. Han J, Flemington C, Houghton AB et al. Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals. Proc.Natl.Acad.Sci.U.S.A. 2001;98:11318-11323.

27. Casale F, Addeo R, D'Angelo V et al. Determination of the in vivo effects of prednisone on Bcl-2 family protein expression in childhood acute lymphoblastic leukemia. Int.J.Oncol. 2003;22:123-128.

28. Findley HW, Jr., Cooper MD, Kim TH, Alvarado C, Ragab AH. Two new acute lymphoblastic leukemia cell lines with early B-cell phenotypes. Blood 1982;60:1305-1309.

29. Strasser-Wozak EMC, Hattmannstorfer R, Hála M et al. Splice site mutation in the glucocorticoid receptor gene causes resistance to glucocorticoid-induced apoptosis in a human acute leukemic cell line. Cancer Res. 1995;55:348-353.

30. Löffler M, Tonko M, Hartmann BL et al. c-myc does not prevent glucocorticoid-induced apoptosis of human leukemic lymphoblasts. Oncogene 1999;18:4626-4631.

31. Pear WS, Nolan GP, Scott ML, Baltimore D. Production of high-titer helper-free retroviruses by transient transfection. Proc.Natl.Acad.Sci.U.S.A 1993;90:8392-8396.

32. Parson W, Kirchebner R, Mühlmann R et al. Cancer cell line identification by short tandem repeat profiling: power and limitations. FASEB J. 2005;19:434-436.

33. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J.Immunol.Methods 1991;139:271-279.

34. Geley S, Hartmann BL, Hattmannstorfer R et al. P53-induced apoptosis in the human T-ALL cell line CCRF-CEM. Oncogene 1997;15:2429-2437.

35. Wu Z, Irizarry RA, Gentleman RC, Martinez Murillo F, Spencer F. A model based background adjustment for oligonucleotide expression arrays. Internet 2004; http://www.bepress.com/jhubiostat/paper1/:

36. Gentleman RC, Carey VJ, Bates DM et al. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 2004;5:R80.

37. Irizarry RA, Bolstad BM, Collin F et al. Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res. 2003;31:e15.

38. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat.Appl.Genet.Mol.Biol. 2004;3:Article3.

39. Dudoit S, Shaffer JP, Boldrick BJ. Multiple hypothesis testing in microarray experiments. Stat.Sci. 2003;18:71-103.

40. Demaison C, Parsley K, Brouns G et al. High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter. Hum.Gene Ther. 2002;13:803-813.

41. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracyclin-responsive promoters. Proc.Natl.Acad.Sci.U.S.A. 1992;89:5547-5551.

42. Kaminski A, Howell MT, Jackson RJ. Initiation of encephalomyocarditis virus RNA translation: the authentic initiation site is not selected by a scanning mechanism. EMBO J. 1990;9:3753-3759.

43. Pepperkok R, Squire A, Geley S, Bastiaens PI. Simultaneous detection of multiple green fluorescent proteins in live cells by fluorescence lifetime imaging microscopy. Curr.Biol. 1999;9:269-272.

44. Ausserlechner MJ, Obexer P, Deutschmann A, Geiger K, Kofler R. A retroviral expression system based on tetracycline-regulated tricistronic transactivator/repressor vectors for functional analyses of antiproliferative and toxic genes. Mol.Cancer Ther. 2006;5:1927-1934.

45. Wu Z, Irizarry RA. Preprocessing of oligonucleotide array data. Nat.Biotechnol. 2004;22:656-658.

46. U M, Shen L, Oshida T et al. Identification of novel direct transcriptional targets of glucocorticoid receptor. Leukemia 2004;18:1850-1856.

47. Vermeer H, Hendriks-Stegeman BI, van der BB, Buul-Offers SC, Jansen M. Glucocorticoid-induced increase in lymphocytic FKBP51 messenger ribonucleic acid expression: a potential marker for glucocorticoid sensitivity, potency, and bioavailability. J.Clin.Endocrinol.Metab 2003;88:277-284.

48. Garcia-Gras EA, Chi P, Thompson EA. Glucocorticoid-mediated destabilization of cyclin D3 mRNA involves RNA-protein interactions in the 3'-untranslated region of the mRNA. J.Biol.Chem. 2000;275:22001-22008.

49. Ausserlechner MJ, Obexer P, Böck G, Geley S, Kofler R. Cyclin D3 and c-myc control glucocorticoid-induced cell cycle arrest but not apoptosis in lymphoblastic leukemia cells. Cell Death Differ. 2004;11:165-174.

50. Czabotar PE, Lee EF, van Delft MF et al. Structural insights into the degradation of Mcl-1 induced by BH3 domains. Proc.Natl.Acad.Sci.U.S.A 2007;104:6217-6222.

51. Willis SN, Chen L, Dewson G et al. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev. 2005;19:1294-1305.

52. Claudio JO, Masih-Khan E, Tang H et al. A molecular compendium of genes expressed in multiple myeloma. Blood 2002;100:2175-2186.

53. Green DR. Apoptotic pathways: ten minutes to dead. Cell 2005;121:671-674.

54. Kim H, Rafiuddin-Shah M, Tu HC et al. Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. Nat.Cell Biol. 2006;8:1348-1358.

55. Willis SN, Fletcher JI, Kaufmann T et al. Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. Science 2007;315:856-859.

56. Willis SN, Adams JM. Life in the balance: how BH3-only proteins induce apoptosis. Curr.Opin.Cell Biol. 2005;17:617-625.

57. Certo M, Del GM, V, Nishino M et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. Cancer Cell 2006;9:351-365.

58. Chen L, Willis SN, Wei A et al. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol.Cell 2005;17:393-403.

59. Villunger A, Michalak EM, Coultas L et al. p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. Science 2003;302:1036-1038.

60. Erlacher M, Michalak EM, Kelly PN et al. BH3-only proteins Puma and Bim are rate-limiting for {gamma} -radiation and glucocorticoid-induced apoptosis of lymphoid cells in vivo. Blood 2005;106:4131-4138.

61. Rhein P, Scheid S, Ratei R et al. Gene expression shift towards normal B cells, decreased proliferative capacity and distinct surface receptors characterize leukemic blasts persisting during induction therapy in childhood acute lymphoblastic leukemia. Leukemia 2007;21:897-905.

62. Alves NL, van Lier RA, Eldering E. Withdrawal symptoms on display: Bcl-2 members under investigation. Trends Immunol. 2007;28:26-32.

63. Herr I, Gassler N, Friess H, Buchler MW. Regulation of differential pro- and anti-apoptotic signaling by glucocorticoids. Apoptosis. 2007;12:271-291.

64. Viegas LR, Vicent GP, Baranao JL, Beato M, Pecci A. Steroid hormones induce bcl-X gene expression through direct activation of distal promoter P4. J.Biol.Chem. 2004;279:9831-9839.

65. Rocha-Viegas L, Vicent GP, Baranao JL, Beato M, Pecci A. Glucocorticoids Repress bcl-X Expression in Lymphoid Cells by Recruiting STAT5B to the P4 Promoter. J.Biol.Chem. 2006;281:33959-33970.

66. Gascoyne DM, Kypta RM, Vivanco MM. Glucocorticoids Inhibit Apoptosis during Fibrosarcoma Development by Transcriptionally Activating Bcl-xL. J.Biol.Chem. 2003;278:18022-18029.

67. Sandau US, Handa RJ. Glucocorticoids exacerbate hypoxia-induced expression of the pro-apoptotic gene Bnip3 in the developing cortex. Neuroscience 2007;144:482-494.

68. Le PP, Friedman JR, Schug J et al. Glucocorticoid Receptor-Dependent Gene Regulatory Networks. PLoS.Genet. 2005;1:e16.

69. Schmidt S, Irving JA, Minto L et al. Glucocorticoid resistance in two key models of acute lymphoblastic leukemia occurs at the level of the glucocorticoid receptor. FASEB J. 2006;20:2600-2602.

70. Wang Z, Chen W, Kono E, Dang T, Garabedian MJ. Modulation of glucocorticoid receptor phosphorylation and transcriptional activity by a C-terminal-associated protein phosphatase. Mol.Endocrinol. 2007;21:625-634.

71. Szatmary Z, Garabedian MJ, Vilcek J. Inhibition of Glucocorticoid Receptor-mediated Transcriptional Activation by p38 Mitogen-activated Protein (MAP) Kinase. J.Biol.Chem. 2004;279:43708-43715.

72. Bodwell JE, Webster JC, Jewell CM et al. Glucocorticoid receptor phosphorylation: Overview, function and cell cycle-dependence. J.Steroid Biochem.Mol.Biol. 1998;65:91-99.

LEGENDS TO FIGURES

Figure 1: Expression and regulation of BCL2 family members in ALL cells

(A) Expression (U133 plus 2.0-derived E-values, log 2 scale) of BCL2 family members in untreated malignant lymphoblasts from 13 children compared to an adult ALL and 2 *in vitro* models (CEM-C7H2, PreB697). An intensity scale is indicated below the graph. E-values and probe sets for this graph are depicted in Table S5. Figure 1B shows U133 plus 2.0-derived regulations of BCL2 family members in peripheral blasts from 13 ALL children at 6-8hs and 24hs of GC mono-therapy[5], and 1C regulations in 6h and 24h GC-treated CEM-C7H2 (top panels) and PreB697 (bottom panels) cells as determined by Exon array-based expression profiling. Extent of regulation (mean M) was plotted against significance (Benjamini-Hochberg adjusted p-values, expressed as negative logarithm to the power of 10). The dotted lines indicate significance of p_{BH}=0.05 and regulation of M= ±1. Genes with p_{BH} values ≤0.05 are indicated. M-values and probe sets for the 6 "volcano plots" are depicted in Tables S6 and S8.

Figure 2: GC-induced apoptosis in CEM-C7H2 and PreB697

CEM-C7H2 and PreB697 ALL cells ($5x10^5$/ml) were cultured in the presence of 100nM dexamethasone or 0.1% as carrier control for the indicated time and extent of apoptosis determined by the propidium iodide method of Nicoletti[33]. The graphs show means of 3 independent experiments ±SD. Aliquots from the same cultures were collected at 6h and 24h to purify RNA for expression profiling.

Figure 3: Expression and GC regulation of BCL2 proteins in CEM-C7H2 T-ALL cells

CCRF-CEM-C7H2 cells were treated with 100nM dexamethasone for 36 hours (A and B) or for the indicated times (C) and analysed by immunoblotting using antibodies specific for the indicated pro- (A) and anti- (B) apoptotic BCL2 proteins. The asterisk marks a recently identified new BMF isoform (Villunger et al., in preparation).

Figure 4: Effect of conditional Bim or BMF knock-down on GC-induced apoptosis

(A) CEM-C7H2-2B10 subclones conditionally expressing shRNA targeting Bim or BMF were cultured for 3d in the presence or absence of 500ng/ml doxycycline (Dox) and subsequently exposed to 100nM dexamethasone (Dex) or 0.1% ethanol as carrier control for up to 72h. Bim and BMF expression was monitored after 24h (Bim) or 30h (BMF) exposure to dexamethasone by immunoblotting using α-tubulin (α-Tub) as loading control. (B) Extent of GC-induced apoptosis was assessed by FACS analysis of propidium iodide-stained nuclei at the times indicated. Bars represent the means ± SD of at least 4 independent experiments.

Figure 5: Effect of conditional BimEL or BMF-1 transgene expression on cell survival

CEM-C7H2-2C8 derivatives conditionally expressing transgenic BimEL (2C8/BimEL#17) or BMF-1 (2C8/BMF1#8) were cultured in the presence of the indicated doxycycline concentrations and analysed by immunoblotting after 3h (A) and by flow cytometry of propidium iodide stained nuclei to determine degree of apoptosis after 24h (B). FACS data shown represent the arithmetic means ± SD of 3 independent experiments.

Figure 6: MCL1 upregulation during GC treatment

(A) 2B10/Bim-shRNA#1 cells pre-cultured in the presence or absence of 500ng/ml doxycycline for 72h were treated with 100nM dexamethasone or 0.1% ethanol for another 24h and analysed

by immunoblotting using antibodies against Bim, MCL1 and α-tubulin as loading control. (B) C7H2-2C8 (left panel) and its derivative 2C8/BimEL#9 (right panel) were treated for the times indicated with 100nM dexamethasone or 12.5ng/ml doxycycline, respectively, and analysed by immunoblotting using antibodies against Bim, MCL1 and α-tubulin. (C) CCRF-CEM-C7H2 cells were pre-treated with 100nM dexamethasone or 0.1% ethanol as control for 24h, then cultured in the presence or absence of 10μM cycloheximide for the indicated times and subjected to immunoblotting with antibodies against MCL1 or α-tubulin as loading control. (D) CCRF-CEM-C7H2 cells were treated with 100nM dexamethasone for 24h and cell lysates were immunoprecipitated with anti-MCL1 antibodies (IP:MCL1) or normal rabbit serum as control (IP: control). Aliquots of immunoprecipitates were subjected to immunoblotting with anti-MCL1 (IB:MCL1) or anti-Bim (IB:BimEL) antibodies.

Figure 7: Effect of knock-down and over-expression of anti-apoptotic BCL2 proteins on GC-induced apoptosis

(A): CEM-C7H2-2B10 (expressing tetR-KRAB) derivatives conditionally expressing shRNAs targeting Bcl-XL (C7H2-2B10/BclX-shRNA-#8 and #13), BCL2 (C7H2-2B10/BCL2-shRNA-#3 and #4) or MCL1 (C7H2-2B10/MCL-shRNA-#6 and #11) were cultured with 500ng/ml doxycycline for the indicated time and analysed by Western blotting with antibodies specific for the indicated BCL2 family members. Shown are data from 1 subclone each. (B): The same cell lines were pre-cultured for 72h in the absence or presence of 500ng/ml doxycycline (Dox), the cultures continued for another 24h with and without dexamethasone (Dex, 100nM, 50nM or 10nM) and apoptosis determined by flow cytometry of propidium iodide-stained nuclei. The data shown represent the arithmetic means ± SD of experiments performed in triplicate with both clones for each gene. (C): CEM-C7H2-2C8 (expressing rtTA) and its derivatives with conditional

expression of transgenic MCL1 (2C8/MCL1#3, #11, #20) were cultured in the absence or presence of 100nM dexamethasone (Dex, 2C8 only) or 100ng/ml doxycycline (Dox, all others) for 24h and MCL1 expression monitored by immunoblotting (left panel). Similarly, Bcl-XL expression was determined in CEM-C7H2 cultured in the absence or presence of 100nM dexamethasone (Dex, C7H2 only) and in its untreated derivatives with constitutive Bcl-XL expression (C7H2/BclXL-2F1, 2F10, and 2G9, right panel). (D): To assess the effect of these proteins on GC-induced apoptosis, MCL1 over-expressing (+Dox) and control (-Dox) cells (left panel) and Bcl-XL over-expressing and parental CEM-C7H2 cells (right panel) were treated with 100nM dexamethasone for the indicated time and analysed by flow cytometry of propidium iodide stained nuclei. Data show means of 3 independent experiments ± SD.

Figure 1: Expression and regulation of BCL2 family members in ALL cells

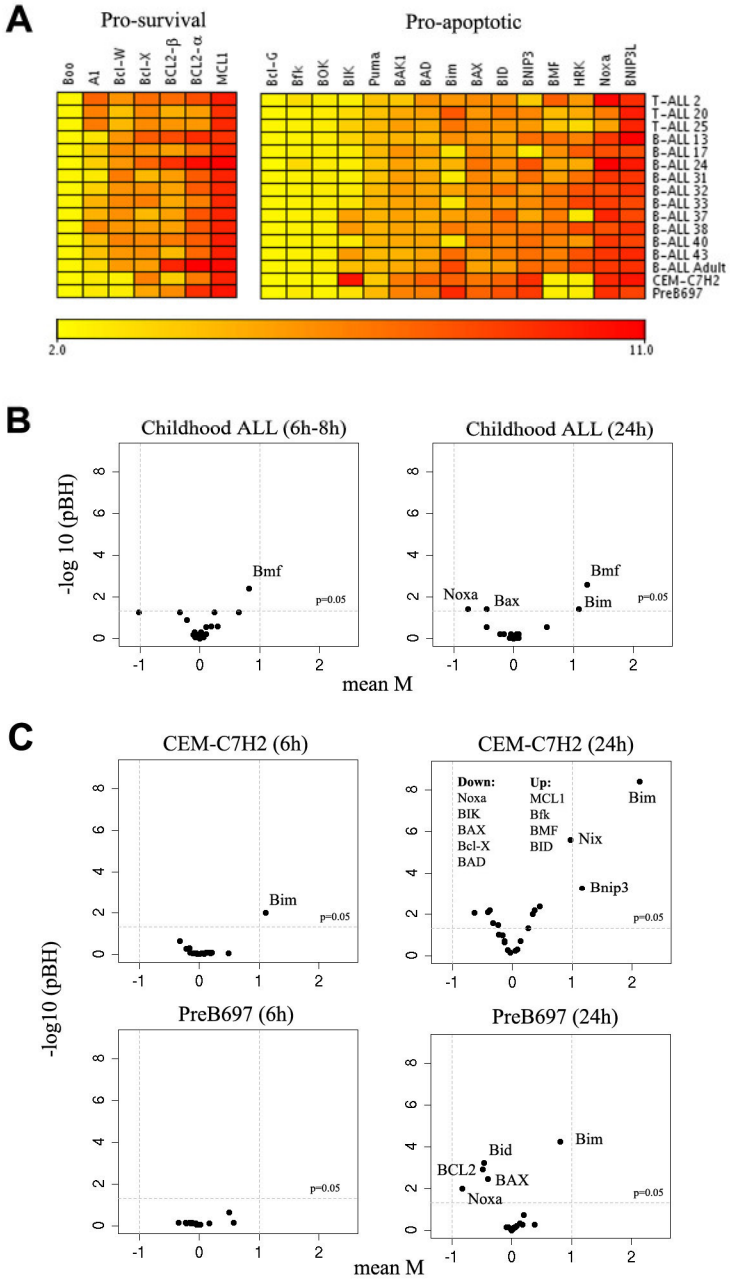


Figure 2: GC-induced apoptosis in CEM-C7H2 and PreB697

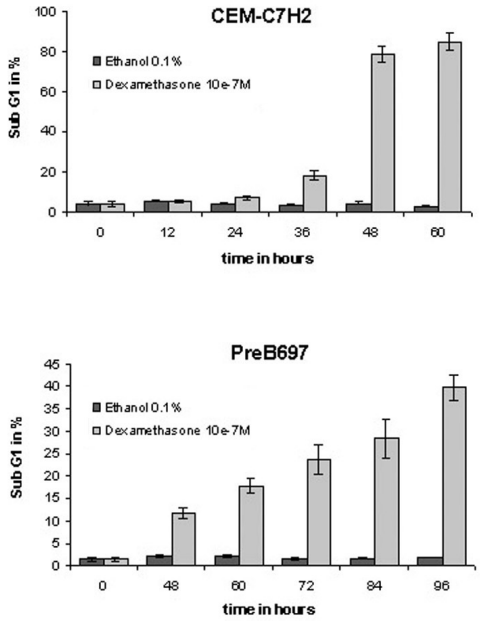


Figure 3: Expression and GC regulation of BCL2 proteins in CEM-C7H2 T-ALL cells

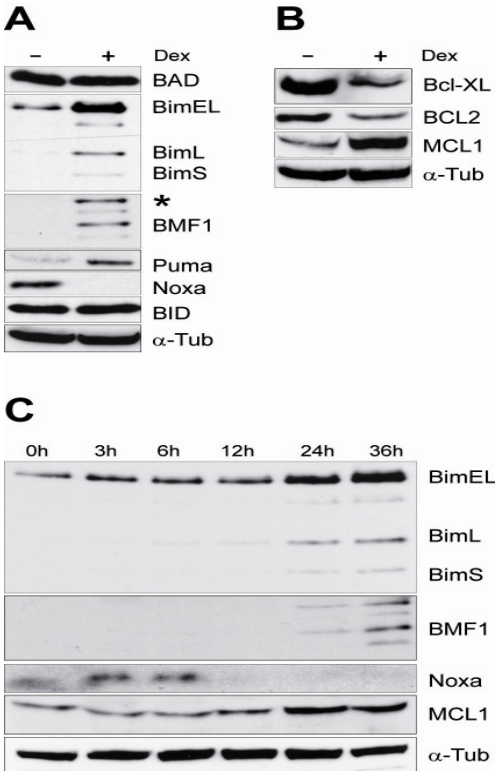


Figure 4: Effect of conditional Bim or BMF knock-down on GC-induced apoptosis

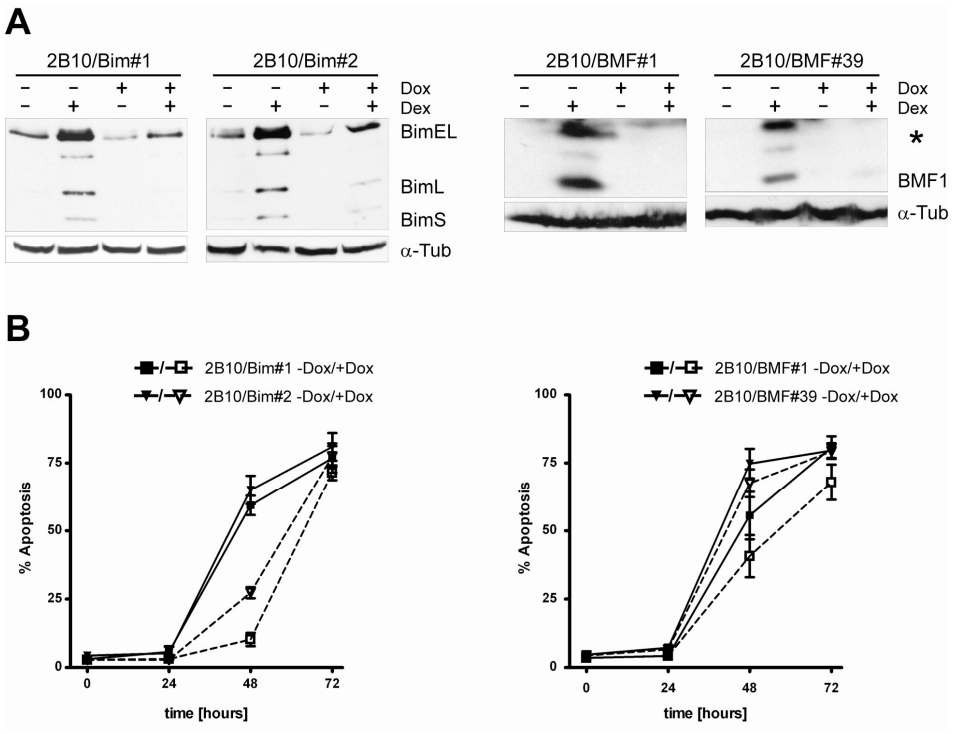


Figure 5: Effect of conditional BimEL or BMF-1 transgene expression on cell survival

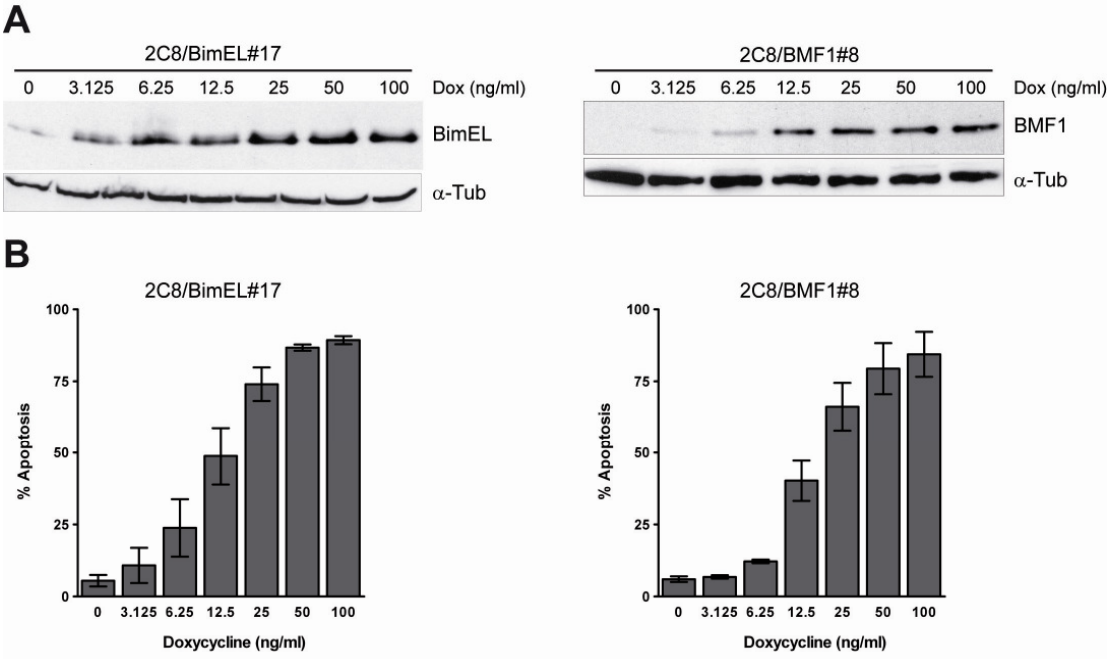


Figure 6: MCL1 upregulation during GC treatment

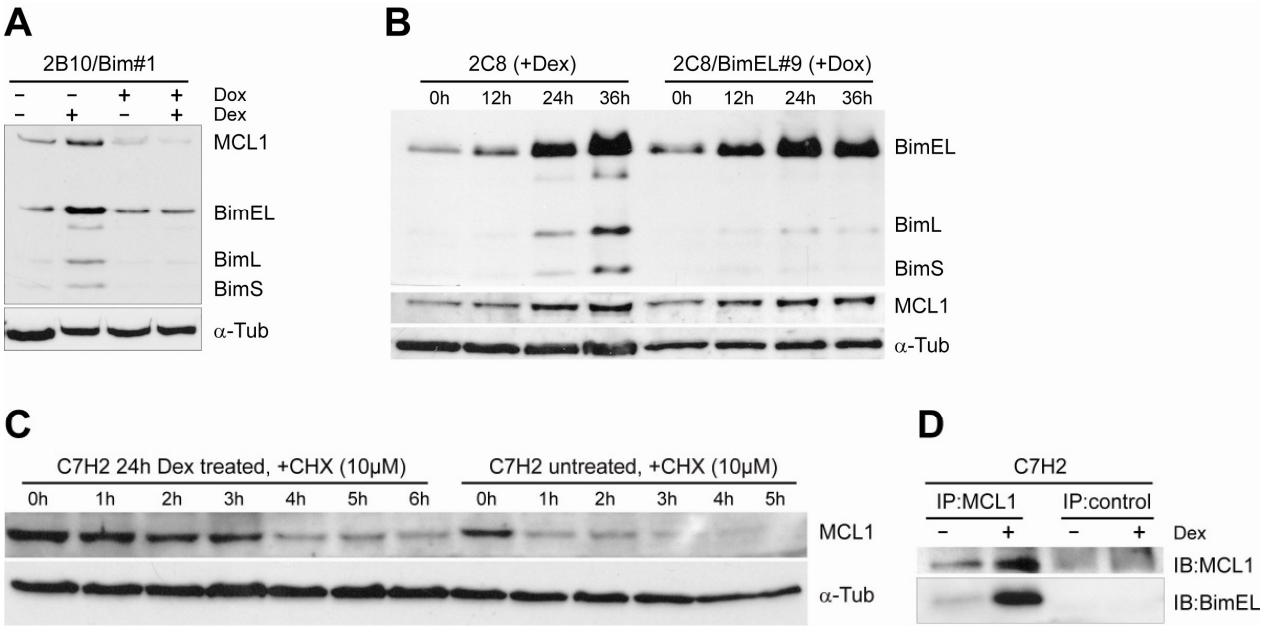
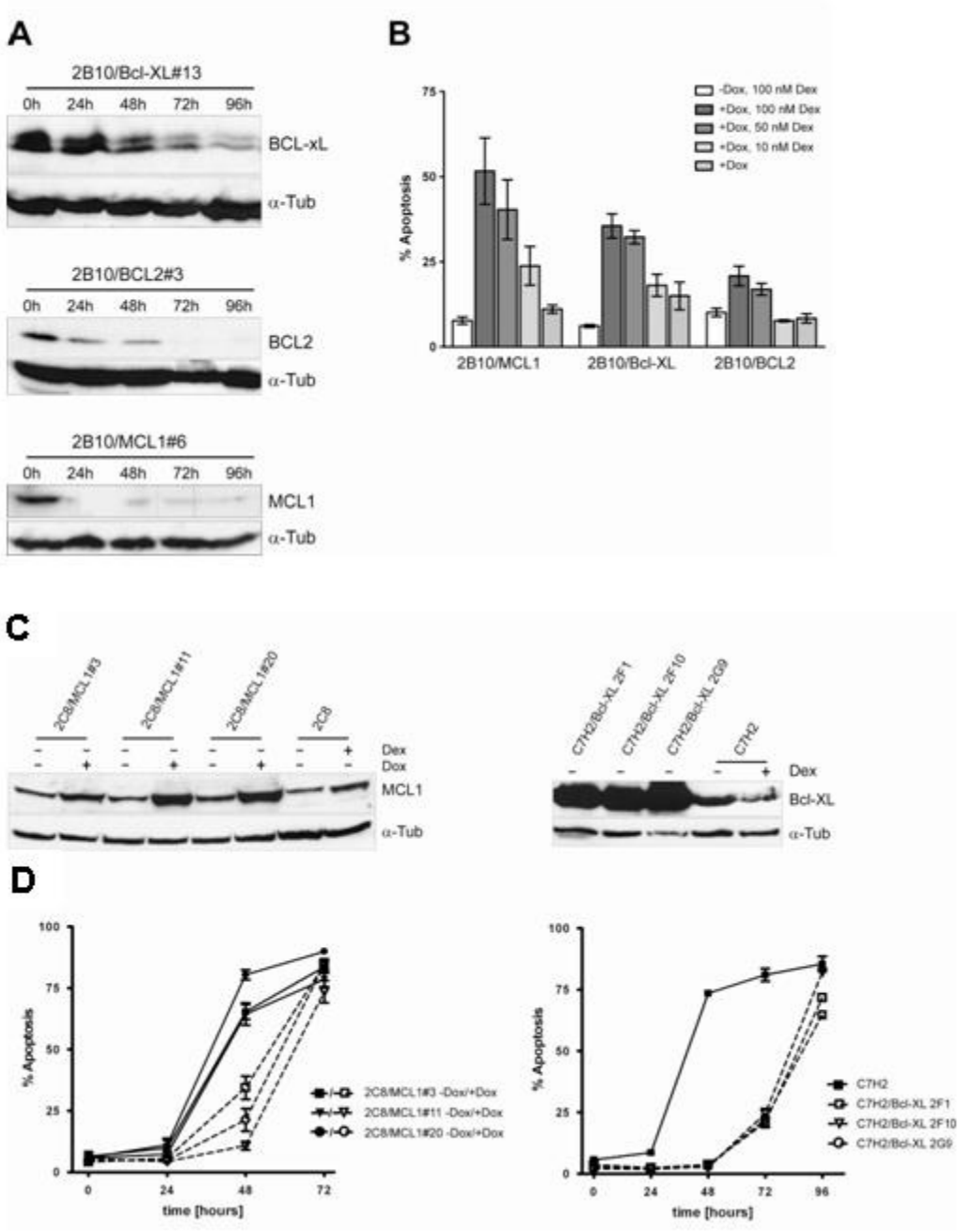


Figure 7: Effect of knock-down and over-expression of anti-apoptotic BCL2 proteins



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CARMAweb: comprehensive R- and bioconductor-based web service for microarray data analysis

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ABSTRACT

CARMAweb (Comprehensive R-based Microarray Analysis web service) is a web application designed for the analysis of microarray data. CARMAweb performs data preprocessing (background correction, quality control and normalization), detection of differentially expressed genes, cluster analysis, dimension reduction and visualization, classification, and Gene Ontology-term analysis. This web application accepts raw data from a variety of imaging software tools for the most widely used microarray platforms: Affymetrix GeneChips, spotted two-color microarrays and Applied Biosystems (ABI) microarrays. R and packages from the Bioconductor project are used as an analytical engine in combination with the R function Sweave, which allows automatic generation of analysis reports. These report files contain all R commands used to perform the analysis and guarantee therefore a maximum transparency and reproducibility for each analysis. The web application is implemented in Java based on the latest J2EE (Java 2 Enterprise Edition) software technology. CARMAweb is freely available at https://carmaweb.genome.tugraz.at.

INTRODUCTION

Expression profiling using microarrays has become a widely used method for the study of gene-expression patterns. Different microarray technologies have become available, including the Affymetrix GeneChip platform (http://www.affymetrix.com), spotted two-color cDNA or oligo microarrays (1), or the ABI single-channel microarrays (Applied Biosystems, http://www.appliedbiosystems.com). All microarray platforms require analytical pipelines with modules for (i) data preprocessing including data normalization, (ii) statistical analysis for identification of differentially expressed genes, (iii) cluster analysis and (iv) Gene Ontology (GO) analysis. The module for normalization and data preprocessing is platform dependent and aims to reduce technical variability without altering the biological variance in the data. After data normalization, the selection of differentially expressed genes is often the main objective of a microarray experiment. Additionally, genes might be grouped into clusters according to the similarity of their expression patterns. Finally, genes can be mapped onto GO (2) terms in order to get an overview of the biological processes, cellular components or molecular functions for which the genes of interest might be involved.

In the past years, Bioconductor (3) (based on the statistical programming language R, http://www.R-project.org) has become the reference tool for the analysis of microarray data because it is based on the most complete set of up-to-date algorithms. However, for scientists without adequate programming experience, the command line usage of R and Bioconductor is too cumbersome. Moreover, the performance of laboratory desktop computers is often insufficient to analyze microarray data with tens of thousands of features. Therefore, many analysis tools with a graphical user interface and powerful computing servers have been developed, including web-based tools like GEPAS (4), ArrayPipe (5), MIDAW (6), RACE (7) or Expression Profiler (8). Of these, only GEPAS and Expression Profiler support both Affymetrix and two-color arrays. To the best of our knowledge, there is currently no web service available for the analysis of the increasingly popular ABI system. MIDAW and RACE use R and Bioconductor packages as analytical engines as well, but these web applications focus either on the analysis of two-color

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microarrays (MIDAW) or Affymetrix GeneChips (RACE). Presently, only Expression Profiler allows loading microarray data from the ArrayExpress database (9). ExpressionProfiler enables direct handling only for raw data from the Affymetrix platform, whereas for two-color microarrays external manipulation of the raw data files has to be performed. The raw data files derived from the image analysis software are usually large and difficult to handle, especially for inexperienced users. Thus, researchers working with two-color microarray data have to navigate several websites and transfer the data between the servers to complete their analyses.

We have therefore developed the web application CARMAweb (Comprehensive R-based Microarray Analysis web service) based on both the latest Java 2 Enterprise Edition (J2EE) software technology and R in combination with Bioconductor.

CARMAweb provides the following unique features:

- Support for Affymetrix, two-color and ABI microarrays,
- Import of raw data from a variety of imaging software tools for two-color microarrays (Agilent Feature Extraction, ArrayVision, BlueFuse, GenePix, ImaGene, QuantArray, SPOT or raw data files from the Stanford Microarray Database),
- A complete analytical pipeline for Affymetrix, two-color and ABI microarrays including modules for preprocessing, detection of differentially expressed genes, clustering and visualization, as well as GO mapping,
- Generation of comprehensive analysis report files.

METHODS

CARMAweb is designed as a multi-tier application based on the J2EE environment, including Java Server Pages and Servlets for the web tier, and Enterprise Java Beans for the middle tier. With the exception of the module for cluster analysis, visualization and classification, all calculations are performed in R using functions of the Bioconductor packages. The connection between Java and R is established through Rserve (http://stats.math.uni-augsburg.de/Rserve/). Each analysis is processed in R using the R function *Sweave* (http://www.ci.tuwien.ac.at/~leisch/Sweave). *Sweave* is a tool that allows embedding of R code into LaTeX documents. *Sweave* executes the R commands from the input file, which is created by the web application. Output from R, R commands and descriptive text are written into a LaTeX file. Thus, code, results and descriptions are presented in a consistent way. After the analysis the LaTeX file generated by *Sweave* is transformed into a pdf analysis report file. These analysis report files contain all R commands used to perform the analysis, together with descriptions for the various methods used. This guarantees a maximum of transparency and reproducibility of each analysis performed in CARMAweb. The CARMAweb user guide gives a short introduction to the various analysis methods available in the web application. Test datasets are provided for each microarray platform.

The current implementation of CARMAweb runs on a server equipped with two AMD Opteron (64 bit CPU) processors and 4 GB of physical memory. CARMAweb will be updated regularly to the newest R and Bioconductor releases. The current version of CARMAweb uses R version 2.2 and Bioconductor release 1.7.

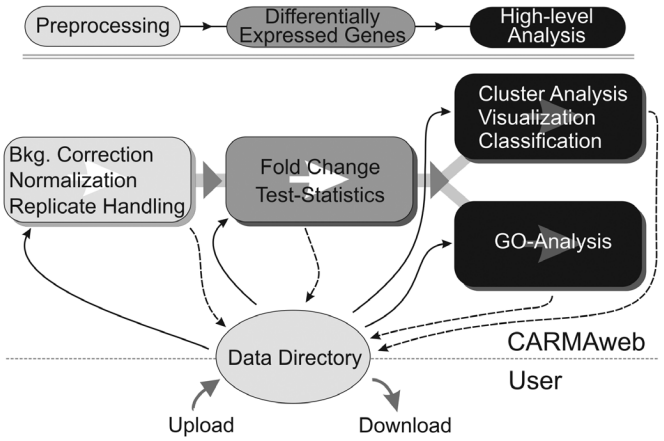

Figure 1. CARMAweb analysis workflow. The different modules of CARMAweb can either be used individually or in combination, resulting in an analytical pipeline. Analysis result files can be returned to the user's data directory and then be used as input for the other modules (e.g. the GO analysis module).

PROGRAM DESCRIPTION

The design and modular conception of CARMAweb allows the use of the different analysis modules either individually or combined into an analytical pipeline (Figure 1). After preprocessing of the raw data and identification of differentially expressed genes, cluster analysis, visualization and GO analysis can be performed. All analysis result files, i.e. tables with normalized expression values, differentially expressed genes or cluster analysis results can be returned to the users' data directory and subsequently used as input files for other analysis modules of CARMAweb or for other applications. Detailed descriptions and help texts for the various processing steps and methods of the different modules are provided as pop-up tool tips.

Data preprocessing

Data preprocessing is an essential step in the analysis of microarray data. The user has to choose an appropriate method from a wide range of available methods depending on the particularities of the data, i.e. their biological characteristics and the platform used.

Two-color microarrays. A large number of image analysis tools is available for two-color microarrays, and several features essential for the data preprocessing (i.e. flags determination, background estimates) differ between them. CARMAweb allows importing raw data files from Agilent Feature Extraction, ArrayVision, BlueFuse, GenePix, ImaGene, QuantArray, SPOT or raw data files from the Stanford Microarray Database. For background correction, CARMAweb allows several options (i) background subtraction, (ii) background subtraction followed by the minimum method (any intensity which is zero or negative after correction is set to half the minimum of the positive corrected intensities), (iii) the moving minimum method (background

estimates are replaced with the minimum of the backgrounds of the spot and its eight neighbors, and are then subtracted from the foreground) or (iv) the method described in (10). After background correction, methods like the median normalization, the loess or print tip loess normalization or the robust spline normalization (normalizes using robustly fitted regression splines and empirical Bayes shrinkage) are provided by CARMAweb to normalize within-array. Afterwards between arrays normalization can be performed using the median scaling or the quantile method. Additionally the variance stabilizing normalization (11), which combines both within and between array normalization, has also been included in CARMAweb. Most of these preprocessing methods are outlined in (12). The preprocessing of two-color microarrays is carried out in CARMAweb using functions from Bioconductors *limma* and *vsn* packages.

Affymetrix GeneChips. Preprocessing of Affymetrix GeneChips generally consists of the following steps: (i) background correction, (ii) normalization, (iii) correction for non-specific binding and (iv) summarization, where the measured probe intensities are averaged to one expression value per probe set. CARMAweb uses the *affy* package from Bioconductor for this purpose, and allows the usage of methods like the Affymetrix MAS5 algorithm or even more sophisticated methods like RMA (robust multi-array average) (13,14) or GCRMA (modified version of RMA that uses probe sequence information for the background correction) to perform the preprocessing. A comparison of the different Affymetrix preprocessing methods is outlined in (15). Additionally it is possible to define custom preprocessing methods by selecting different algorithms for each one of the preprocessing steps. For Affymetrix GeneChip analyses, Affymetrix raw data files (CEL files) are used as input files.

ABI microarrays. The module for the ABI microarray preprocessing supports tabulator-delimited text files, which can be exported from ABIs scanning software. These text files already contain background-corrected expression values from one or more microarrays. CARMAweb permits reading of microarray data from one or more of such exported text files, and allows the adjustment of raw (background-corrected) expression values across all microarrays of one experiment using quantile normalization. Alternatively, the assay-normalized signal provided by ABI might be used for the analysis (see Applied Biosystems 1700 Chemiluminescent Microarray Analyzer User's manual http://www.appliedbiosystems.com). Quality parameters (flags, signal to noise, cv) can be used to filter out poor quality spots.

Following the microarray preprocessing, replicated arrays can be averaged in an optional replicate handling step. This function also allows averaging of the replicated spots within each microarray, and its aim is to increase the quality of the microarray data by reducing the noise.

Detection of differentially expressed genes

The detection of differentially expressed genes can be performed in CARMAweb for microarray experiments with a small number of biological replicates using a simple fold change. Additionally CARMAweb allows ranking of genes according to the number of comparisons in which they were selected as differentially expressed.

In microarray experiments with a sufficient number of arrays, differentially expressed genes can be detected in CARMAweb using statistical tests like the Mann Whitney U test (16), the Student's *t*-test (16), the permutation (randomization) test (16), the moderated *t*-statistics (based on an empirical Bayes approach, the Bioconductors *limma* package) (17) or the significance analysis of microarrays (SAM, Bioconductors *siggenes* package) (18). Because microarray experiments generate large multiplicity problems in which thousands of hypotheses are tested simultaneously within one experiment (19), an adjustment of the calculated *P*-values should be performed. Bioconductors *multtest* package provides suitable methods to adjust *P*-values regarding this multiple hypothesis testing problem. Available adjustment methods are the procedure introduced by Benjamini and Hochberg (20) for strong control of the FDR (false discovery rate, expected proportion of false positives among the rejected hypotheses) or the method by Westfall and Young (21) to control the FWER (family-wise error rate, probability of at least one false positive). CARMAweb allows the use of all methods described in (19) for the adjustment of raw *P*-values.

To alleviate the loss of power from the formidable multiplicity of gene-by-gene hypothesis testing within a microarray experiment, a non-specific pre-filtering of the data can also be performed. This pre-filtering consists in the reduction beforehand of the number of genes to be tested, removing those that are either not relevant for the study in question or those expected to be unaltered through the experimental conditions. This can be achieved by focusing the analysis only on those genes for which variance across conditions is in the top $x\%$, where x is a user-defined value.

Cluster analysis, dimension reduction and visualization, and classification

For cluster analysis, dimension reduction and visualization, and for classification of microarray data, the module GenesisWeb can be used (Figure 2). This module is based on the cluster analysis suite Genesis (22), and uses its server (23) to perform the calculations. Cluster analysis and visualization requires intensive graphical user interaction that is not supported by R. The cluster analysis module of CARMAweb supports an interactive selection, coloring and export of clusters, and also displays other important information like the expression values or gene names both as tool tips and in the status bar of the browser when the user moves the mouse over the image.

Expression data can be adjusted beforehand with methods like mean or median centering, logarithmic transformation or division by the SD across samples or genes. Genes and/or samples can be grouped according to their expression similarity using the hierarchical clustering algorithm (HCL) (24), the *k*-means clustering method (KMC) (25) or self-organizing maps (SOM) (26). A wide range of distance-measurement methods is available to measure the similarity of gene or sample expression patterns (e.g. Euclidian distance, Pearson correlation, Spearman's rank or Kendall's tau). As mentioned before all result images are interactive, thus allowing the selection, coloring and export of clusters. Additional information, like gene names or expression values, is displayed both as

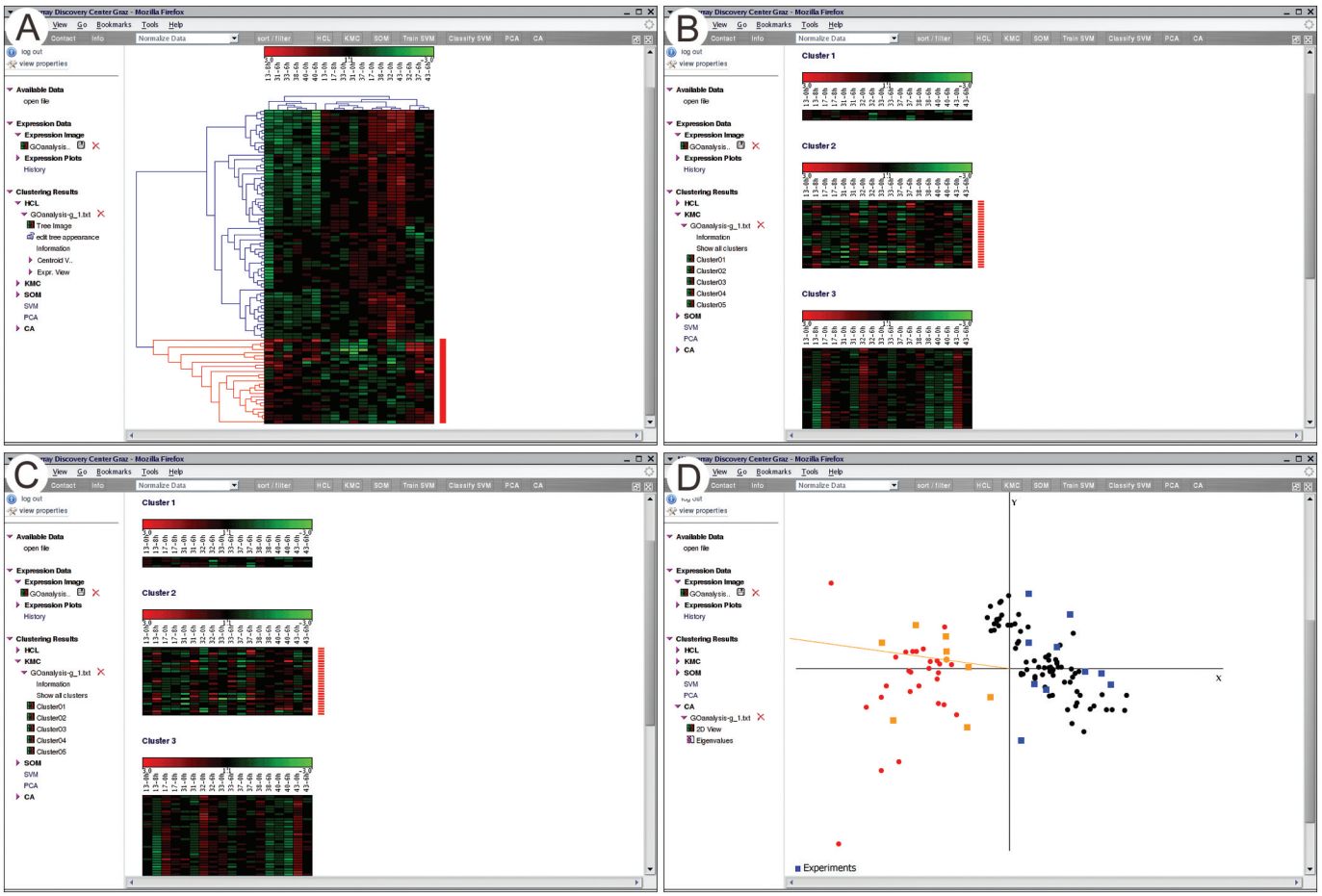

Figure 2. The cluster analysis module GenesisWeb offers interactive cluster selection. (**A**) Result from a hierarchical cluster analysis. (**B**) Result from *k*-means cluster analysis of the same dataset. (**C**) Result from SOM cluster analysis. (**D**) Visualization of a CA of the same dataset. Clusters interactively selected in any of the cluster analyses can be highlighted in further analyses (shown here as red labeled genes).

tool tips and in the status bar of the browser when the user moves the mouse over expression or cluster images.

The available dimension reduction and visualization methods are principal component analysis (PCA) (27) and correspondence analysis (CA) (28). Whereas PCA can be used to identify key variables (or combination of variables) in the datasets, CA allows simultaneous detection of dependencies between samples and genes in microarray data . Visualization tools available with the dimension reduction procedure also enable selection, coloring and export of genes that group together in the space spanned by the principal components.

Support vector machines (SVM) (29) can be used for classification of microarray data. The aim of this supervised classification method is to classify genes or samples by using the information gathered from the training on a dataset with known classification. For example, an SVM can learn in the training step what expression features are specific for a given functional group of genes specified by the researcher, and use this information to decide whether any given gene is likely to be a member of the group or not.

The tool can be used as a standalone web application at https://carmaweb.genome.tugraz.at/genesis, or in combination with CARMAweb, where it is possible to return cluster analysis results to the user's data directory. As input files, tabulator-delimited text files containing expression values (e.g. from an earlier analysis that detected differentially expressed genes, or from files uploaded by the user) are supported.

GO analysis

The Gene Ontology project (2) provides three independent ontologies for gene products. The three ontologies refer to the cellular component, biological process and molecular function of a gene product and allow its description in a hierarchical manner. The GO analysis aims to assist in the biological interpretation of the results by finding GO terms that are significantly often associated to genes in a given gene list. CARMAweb uses the *GOstats* and *GO* package from Bioconductor for the GO term analysis. The GO term analysis module of CARMAweb supports as input any tabulator-delimited text file that contains one column with EntrezGene (formerly LocusLink) identifiers of the genes of interest. This kind of input file can be either file uploaded by the user or result file from a previous analysis. The result of the GO analysis is a GO graph, and a table with GO terms and *P*-values calculated for over-representation of the genes in the corresponding GO terms. The GO graph is the collection of unique GO terms

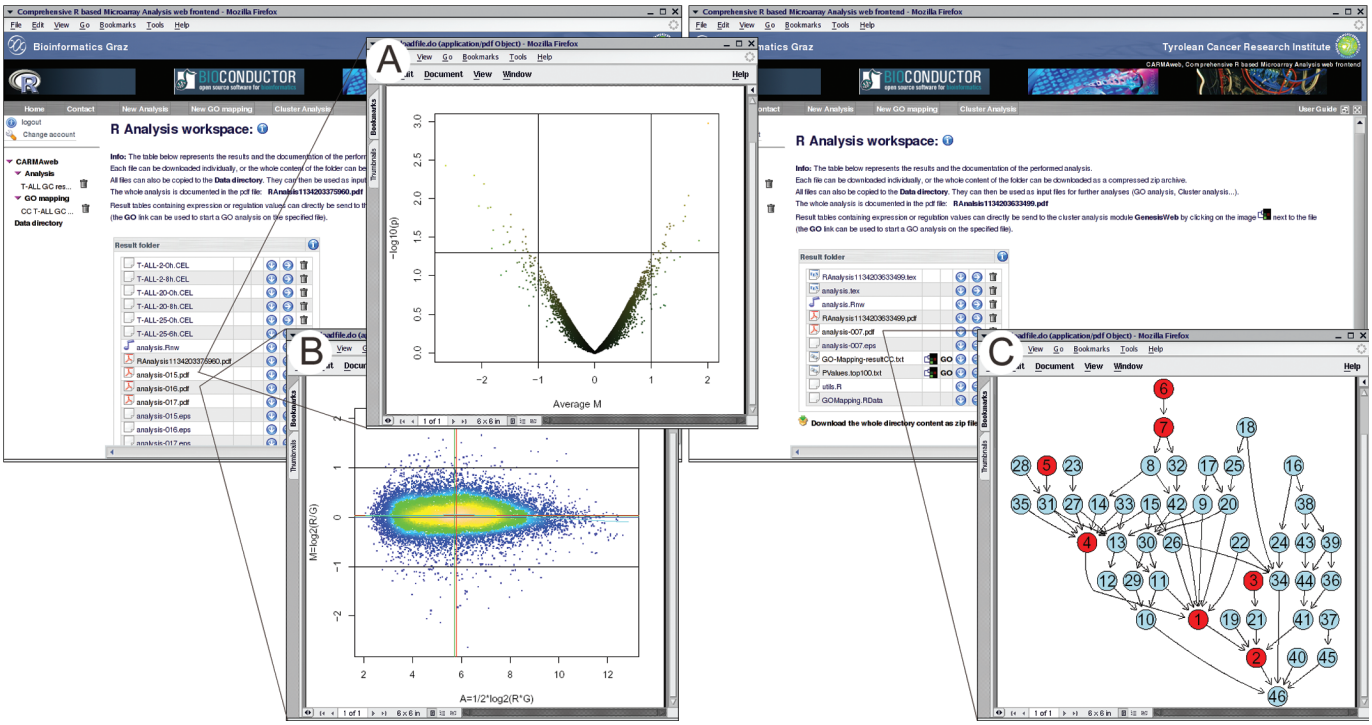

Figure 3. Result workspaces of a differentially expressed genes analysis (left) and a GO analysis (right). (**A**) Volcano plot displaying the mean differential expression against *P*-values (−log10 of the *P*-value) of all genes. (**B**) MA plot. Points are colored according to local point density with brighter colors coding for higher density. (**C**) The induced GO graph of the genes of interest. Red nodes represent over-represented GO terms.

that are associated with one or more of the genes of interest. In order to allow calculation of *P*-values, an additional file containing the EntrezGene identifiers of all genes that can be detected with the microarrays in use needs to be submitted. Affymetrix users can specify the GeneChip used in the analysis instead of submitting a file with all EntrezGene identifiers. Although some correction for multiple testing should be performed on the *P*-values, such tests are not independent and the sampling distribution is unclear (30), so CARMAweb at present does not perform any correction.

Output and analysis results

Each analysis is processed in its own workspace, which is accessible only to the user performing the analysis (Figure 3). The analysis result includes all raw data files, the analysis report file containing all commands and descriptions about the methods used, and all result tables and plots created during the analysis. Additionally the R workspace of each analysis step can be exported to an *RData* file, which can be used to continue the analysis in R on a local workstation. The result tables can comprise tables of normalized expression values for all genes in all arrays, tables with expression values of the subsets of differentially expressed genes, or tables containing the raw *P*-values and adjusted *P*-values using the various adjustment methods. In an Affymetrix GeneChip analysis all probe sets are annotated to the identifiers of the publicly available databases (GenBank (31), UniGene, EntrezGene) using the Bioconductor *annaffy* package. Analysis result files can be returned to the users' data directory and be used as input for further analyses.

Visualizations of the microarray data like MA plots, histograms, box plots or volcano plots are available as single files and are additionally embedded into the analysis report file. The content of each analysis workspace can be downloaded after completion as a single zip archive, or each file can be downloaded separately. The GO term analysis produces a directed acyclic graph of all GO terms to which the genes of interest are associated (Figure 3). Additionally a table containing all GO terms with the corresponding *P*-value is created. The *P*-values provide information about the over-representation of the genes of interest to the term compared with the total number of genes associated with it. The table contains the number of genes of interest that are mapped to the specific term and the total number of genes present on the microarray in use that are associated with the GO term.

Future development

The next release of CARMAweb will provide a complete SOAP (Simple Object Access Protocol) interface to its analysis facilities, thus allowing other applications to use the analysis and processing steps available in CARMAweb.

CONCLUSIONS

The web application CARMAweb that we have developed combines the advantages of an intuitive web-based graphical user interface with the wide range of state-of-the-art microarray normalization and analysis methods provided by Bioconductor. Owing to the modular structure of CARMAweb and the standards-based software engineering, extensions or new

functionalities can be implemented easily without complex and time-consuming alterations of the source code.

CARMAweb provides several unique features in a modular and flexible system for the analysis of microarray data. First, data from three platforms, namely Affymetrix GeneChip, two-color microarray and the ABI microarray platform can be analyzed. Second, a wide range of file formats for two-color microarray raw data is supported. Third, a complete analytical pipeline for the supported platforms is provided, including preprocessing, detection of differentially expressed genes, cluster analysis, dimensionality reduction and visualization, classification, and GO analysis. Fourth, data exploration is enhanced by analysis report files that include the parameters and commands used. The report files that are generated specifically for each analysis guarantee a maximum of transparency and reproducibility. Furthermore, these report files provide a unique way for the documentation of any analyses that have been performed by recording how and with which methods the analysis results have been derived. In sum, based on its flexibility in selecting different analysis steps, its possibility for customization and its comprehensive web-based graphical user interface, CARMAweb is a powerful tool for microarray data analysis.

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Conflict of interest statement. None declared.

REFERENCES

1. Schena,M., Shalon,D., Davis,R.W. and Brown,P.O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science*, **270**, 467–470.
2. Gene Ontology Consortium. (2001), Creating the gene ontology resource: design and implementation. *Genome Res.*, **11**, 1425–1433.
3. Gentleman,R.C., Carey,V.J., Bates,D.M., Bolstad,B., Dettling,M., Dudoit,S., Ellis,B., Gautier,L., Ge,Y., Gentry,J. *et al.* (2004) Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.*, **5**, R80.
4. Herrero,J., Al-Shahrour,F., Diaz-Uriarte,R., Mateos,A., Vaquerizas,J.M., Santoyo,J. and Dopazo,J. (2003) GEPAS: a web-based resource for microarray gene expression data analysis. *Nucleic Acids Res.*, **31**, 3461–3467.
5. Hokamp,K., Roche,F.M., Acab,M., Rousseau,M.-E., Kuo,B., Goode,D., Aeschliman,D., Bryan,J., Babiuk,L.A., Hancock,R.E.W. *et al.* (2004) ArrayPipe: a flexible processing pipeline for microarray data. *Nucleic Acids Res.*, **32**, W457–W459.
6. Romualdi,C., Vitulo,N., Del Favero,M. and Lanfranchi,G. (2005) MIDAW: a web tool for statistical analysis of microarray data. *Nucleic Acids Res.*, **33**, W644–W649.
7. Psarros,M., Heber,S., Sick,M., Thoppae,G., Harshman,K. and Sick,B. (2005) RACE: Remote Analysis Computation for gene Expression data. *Nucleic Acids Res.*, **33**, W638–W643.
8. Kapushesky,M., Kemmeren,P., Culhane,A.C., Durinck,S., Ihmels,J., Korner,C., Kull,M., Torrente,A., Sarkans,U., Vilo,J. *et al.* (2004) Expression Profiler: next generation—an online platform for analysis of microarray data. *Nucleic Acids Res.*, **32**, W465–W470.
9. Brazma,A., Parkinson,H., Sarkans,U., Shojatalab,M., Vilo,J., Abeygunawardena,N., Holloway,E., Kapushesky,M., Kemmeren,P., Lara,G.G. *et al.* (2003) ArrayExpress—a public repository for microarray gene expression data at the EBI. *Nucleic Acids Res.*, **31**, 68–71.
10. Edwards,D.E. (2003) Non-linear normalization and background correction in one-channel cDNA microarray studies. *Bioinformatics*, **19**, 825–833.
11. Huber,W., von Heydebreck,A., Sultmann,H., Poustka,A. and Vingron,M. (2002) Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics*, **18**, 96–104.
12. Smyth,G.K. and Speed,T.P. (2003) Normalization of cDNA microarray data. *Methods*, **31**, 265–273.
13. Irizarry,R.A., Hobbs,B., Collin,F., Beazer-Barclay,Y.D., Antonellis,K.J., Scherf,U. and Speed,T.P. (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*, **4**, 249–264.
14. Irizarry,R.A., Bolstad,B.M., Collin,F., Cope,L.M., Hobbs,B. and Speed,T.P. (2003) Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res.*, **31**, e15.
15. Bolstad,B.M., Irizarry,R.A., Astrand,M. and Speed,T.P. (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics*, **19**, 185–193.
16. Motulsky,H. (1995) *Intuitive Biostatistics*. Oxford University Press, Oxford.
17. Smyth,G.K., Yang,Y.H. and Speed,T. (2003) Statistical issues in cDNA microarray data anlalysis. *Methods Mol. Biol.*, **224**, 111–136.
18. Tusher,V., Tibshirani,R. and Chu,G. (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl Acad. Sci. USA*, **98**, 5116–5124.
19. Dudoit,S., Shaffer,J.P. and Boldrick,B.J. (2003) Multiple hypothesis testing in microarray experiments. *Stat. Sci.*, **18**, 71–103.
20. Benjamini,Y. and Hochberg,Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser.*, **57**, 289–300.
21. Westfall,P.H. and Young,S. (1993) *Resampling-Based Multiple Testing: Examples and Methods for P-Value Adjustment*. Wiley, NY.
22. Sturn,A., Quackenbush,J. and Trajanoski,Z. (2002) Genesis: cluster analysis of microarray data. *Bioinformatics*, **18**, 207–208.
23. Sturn,A., Mlecnik,B., Pieler,R., Rainer,J., Truskaller,T. and Trajanoski,Z. (2003) Client-server environment for high-performance gene expression data analysis. *Bioinformatics*, **19**, 772–773.
24. Eisen,M.B., Spellman,P.T., Brown,P.O. and Botstein,D. (1998) Cluster analysis and display of genome-wide expression patterns. *Proc. Natl Acad. Sci. USA*, **95**, 14863–14868.
25. Tavazoie,S., Hughes,J.D., Campbell,M.J., Cho,R.J. and Church,G.M. (1999) Systematic determination of genetic network architecture. *Nature Genet.*, **22**, 281–285.
26. Tamayo,P., Slonim,D., Mesirov,J., Zhu,Q., Kitareewan,S., Dmitrovsky,E., Lander,E.S. and Golub,T.R. (1999) Interpreting patterns of gene expression with self-organizing maps: methods and application to hematopoietic di erentiation. *Proc. Natl Acad. Sci. USA*, **96**, 2907–2912.
27. Yeung,K.Y. and Ruzzo,W.L. (2001) Principal component analysis for clustering gene expression data. *Bioinformatics*, **17**, 763–774.
28. Fellenberg,K., Hauser,N.C., Brors,B., Neutzner,A., Hoheisel,J.D. and Vingron,M. (2001) Correspondence analysis applied to microarray data. *Proc. Natl Acad. Sci. USA*, **98**, 10781–10786.
29. Brown,M.P., Grundy,W.N., Lin,D., Cristianini,N., Sugnet,C.W., Furey,T.S., Ares,M.J. and Haussler,D. (2000) Knowledge-based analysis of microarray gene expression data by using support vector machines. *Proc. Natl Acad. Sci. USA*, **97**, 262–267.
30. Gentleman,R., Scholtens,D., Ding,B., Carey,V.J. and Huber,W. (2005) Case Studies Using Graphs on Biological Data. In Gentleman,R., Carey,V.J., Huber,W., Irizarry,R.A. and Dudoit,S. (eds), *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*. Springer, NY, pp. 375–378.
31. Benson,D.A., Karsch-Mizrachi,I., Lipman,D.J., Ostell,J. and Wheeler,D.L. (2005) GenBank. *Nucleic Acids Res.*, **33**, 34–38.

Identification of glucocorticoid-response genes in children with acute lymphoblastic leukemia

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The ability of glucocorticoids (GCs) to kill lymphoid cells led to their inclusion in essentially all chemotherapy protocols for lymphoid malignancies, particularly childhood acute lymphoblastic leukemia (ALL). GCs mediate apoptosis via their cognate receptor and subsequent alterations in gene expression. Previous investigations, including expression profiling studies with subgenome microarrays in model systems, have led to a number of attractive, but conflicting, hypotheses that have never been tested in a clinical setting. Here, we present a comparative whole-genome expression profiling approach using lymphoblasts (purified at 3 time points) from 13 GC-sensitive children undergoing therapy for ALL. For comparisons, expression profiles were generated from an adult patient with ALL, peripheral blood lymphocytes from GC-exposed healthy donors, GC-sensitive and -resistant ALL cell lines, and mouse thymocytes treated with GCs in vivo and in vitro. This generated an essentially complete list of GC-regulated candidate genes in clinical settings and experimental systems, allowing immediate analysis of any gene for its potential significance to GC-induced apoptosis. Our analysis argued against most of the model-based hypotheses and instead identified a small number of novel candidate genes, including *PFKFB2,* a key regulator of glucose metabolism; *ZBTB16,* a putative transcription factor; and *SNF1LK,* a protein kinase implicated in cell-cycle regulation. (Blood. 2006;107:2061-2069)

Introduction

Glucocorticoid (GC)–induced apoptosis is a phenomenon of considerable physiologic and therapeutic significance. Physiologically, it has been implicated in the shaping of the immune repertoire and in controlling immune responses,[1] and therapeutically it has been exploited in the treatment of lymphoid malignancies, most notably childhood acute lymphoblastic leukemia (ALL),[2] where good response to introductory hormone treatment predicts favorable overall outcome.[3] Thus, defining the molecular basis of GC-induced cell death[4-7] and the clinically relevant phenomenon of GC resistance[8-12] has obvious bearing on understanding immune system regulation and developing improved therapy protocols for lymphoid malignancies.

GCs mediate most of their effects via their cognate receptor (GC receptor [GR]), a ligand-activated transcription factor of the large nuclear receptor family.[13] GC-induced apoptosis critically depends on sufficient levels of GRs and subsequent alterations in gene expression, but the precise nature of the GC-regulated genes responsible for the antileukemic GC effects remains elusive.[4-7] To address this issue, we and others performed expression profiling with subgenome microarrays (up to ~ 10 000 genes) and various model systems of GC-induced cell death (mouse and human leukemia cell lines and mouse thymocytes). These studies identified a large number of GC-regulated genes and led to several hypotheses (reviewed in Schmidt et al[4]). Specifically, GCs may induce cell death by directly regulating the expression of components of the cell death machinery, such as components of the intrinsic pathway, including the Bcl-2 rheostat;[14] the extrinsic pathway, comprising membrane death receptors and their signaling proteins;[15,16] or the effector molecules of the death machinery (ie, the caspases).[17,18] In support of this theory, transcriptional induction of the Bcl-2 homology 3 (BH3)–only molecule Bim,[19] the caspase-activating granzyme A,[20] or a potentially proapoptotic molecule, called *GPR65/TDAG8,*[21] have been suggested to cause GC-induced apoptosis. Alternatively, GCs may deregulate cellular homeostasis, which, in turn, is interpreted by the cell as

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S. Schmidt participated in most experiments, coordinated the data analyses, and contributed to the writing of the manuscript; J.R. performed the bioinformatic data analyses; S.R. generated the GR^{dim} transfected cell lines; C.P. coordinated and performed the mouse work; S.J. performed the microarray analyses; C.A., E.P., S. Skvortsov, and R.C. were involved in patient sample preparation and analyses; M.F. coordinated the adult ALL data; T.R. and O.A.J. performed and analyzed the GBA; S.G. assisted in writing and data interpretation; B.M. coordinated the clinical work with children; and R.K. coordinated the entire study and contributed to the writing of the manuscript. The final version was seen and approved by all authors.

The online version of the article contains a data supplement.

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a death signal and subsequently triggers the apoptotic response. As one controversial example,[22,23] GC repression of c-myc has been proposed to generate a "conflicting signal" that is not tolerated by proliferating leukemia cells and activates a cell death program. Other proposed examples include GC-mediated deregulation of metabolism[24] and/or macromolecule neosynthesis.[25] These GC effects may be critically enhanced by GR autoinduction, which is observed in several models of GC-induced apoptosis, but not in tissues that do not undergo cell death upon GC exposure (reviewed in Kofler[26]). Very recently, a weak, but significant, induction of mitogen-activated protein kinase (MAPK) kinase 3 was suggested to contribute to GC sensitivity by activating p38 MAPK which, in turn, phosphorylates the GR, thereby increasing its transactivation potential.[27] However, whether any of these hypotheses can be extended to the clinical setting has not been investigated.

In this study, we addressed the molecular basis of GC-induced apoptosis by a novel comparative expression profiling strategy that used children with ALL and several other biological systems of GC sensitivity and resistance (Figure 1). First, we determined the genes regulated in malignant lymphoblasts from 13 GC-sensitive children with ALL during GC monotherapy using Affymetrix-based "whole-genome" expression profiling (Affymetrix, Santa Clara, CA). This database (Database 1 in Figure 1) can be used to query essentially any gene in the genome for in vivo regulation in ALL, a prerequisite for a potential upstream GC response gene in this death pathway. In a second step, we determined the genes that were coordinately regulated in most patients ("Initial candidates list"), followed by enrichment for early responding genes. The remaining 49 genes should include the upstream component(s) of a putative canonical pathway to GC-induced apoptosis mingled with genes not involved in the death response. To further address this issue, we determined the expression profiles of several additional biological systems in which GCs do or do not induce cell death (Database 2 in Figure 1). As explained in Table 1, this database provides evidence for or against a possible role in GC-induced apoptosis of response genes identified in childhood patients with ALL, and aided in both identifying a set of genes unlikely to be directly regulated by GC (cell-cycle genes; Figure 1) and in evaluating the significance of the final list of most probable candidates (Table 5). In conclusion, our study provides new and essential insight into the GC response genes and possible molecular mechanism of GC-induced apoptosis in essentially all relevant biological systems, most importantly, children with ALL.

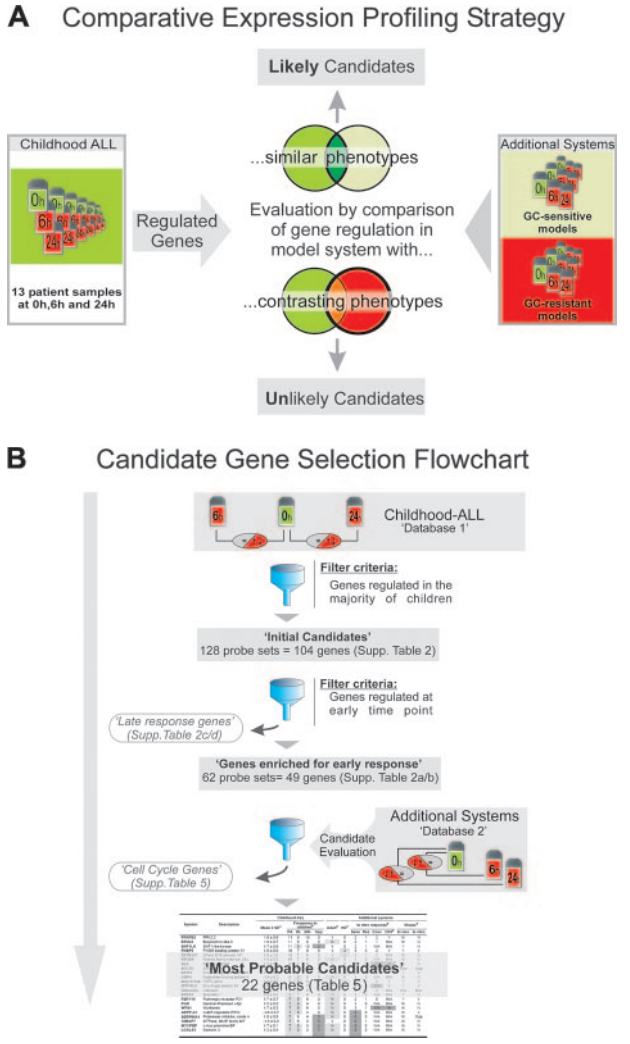

Figure 1. Comparative expression profiling strategy and general work flow. (A) The principle of comparative expression profiling exemplified by evaluating candidate genes (ie, genes regulated in the majority of childhood ALL samples) in additional GC-sensitive and GC-resistant systems. Coregulation in the former supports, whereas coregulation in the latter argues against, a potential role of a candidate gene in the death response. Other relevant information, such as interspecies conservation, de novo protein biosynthesis-dependence, etc, can also be derived by comparisons with the additional systems as outlined in Table 1. (B) Summary of workflow described in "Introduction." The complete databases are available at www.ncbi.nlm.nih.gov/geo/ (GEO accession numbers: GSE2677, GSE2842, GSE2843). Tables corresponding to various database subsets are shown in the Supplemental Materials, as indicated. The final 22 genes are presented in Table 5. The additional systems comprising database 2 and their use for evaluating the possible significance of the candidate genes in the death response are explained in Table 1.

Patients, materials, and methods

Patients and other biologic systems

Patients. Children with ALL admitted to the Department of Pediatrics, Innsbruck Medical University, and treated according to Berlin-Frankfurt-Münster (BFM) protocol 2000 were enrolled in this study. The study was approved by the Ethics Committee of the Innsbruck Medical University (EK1-1193-172/35) and written informed consent was obtained from parents or custodians. For comparison purposes, a 72-year-old white male with B-cell precursor (BCP)–ALL treated at the Department of Hematology and Oncology of the Innsbruck Medical University was included after giving written informed consent.

Blood sampling, GC treatment, and GC response. EDTA blood was taken by venous puncture prior to initiation of GC treatment, and at 6- to 8-hour intervals after initiation. To avoid tumor lysis syndrome, the daily GC dose was gradually increased over the first 3 to 4 days to reach 60 mg prednisolone/m^2/day. Treatment was initiated with a single intravenous or oral application of 6% to 38% of the final dose, depending on peripheral blast counts, T- or B-cell phenotype, and clinical conditions. On the second day, the children received 30% to 60% of the final GC dose in 3 applications. To account for treatment differences, GC bioactivity was determined in the sera (Table 1). The adult patient received a single oral application of 20 mg dexamethasone on day 1 and another 12 mg on the morning of day 2. All patients responded to the treatment with a reduction of peripheral lymphoblasts within

Table 1. Use of additional biological systems for candidate gene evaluation

Biological system	Major information	Coregulation	
		Yes	No
Adult ALL in vivo*	Exclusion of MTX effects; identification of genes coregulated in childhood and adult GC-sensitive ALL	+	−
BCP-ALL-40 ex vivo	Identification of cell autonomous GC effects in primary leukemia cells	+	−
PBL in vivo, GC resistant	Regulated genes do not directly induce cell death	−	+
GC-sensitive ALL cell lines	Coregulation supports cell autonomous effects, GC specificity, and possible role in the death response	+	−
GC-resistant ALL cell lines†	Loss of coregulation compared to sensitive counterpart supports role in death response	−	+
Cell lines with restored GC sensitivity‡	Restoration of loss of coregulation strongly supports role in death response	+	−
Mouse thymocytes§	Identification of interspecies conserved response genes	+	−
CHX-treated ALL cell line‖	Dependence on de novo protein synthesis excludes primary response genes	+	−

Whole genome expression profiles were obtained from the systems listed prior to and after exposure to GC in vivo or in vitro, as indicated. Performance of a candidate gene in these systems (ie, absence/presence and/or extent of GC regulation) provides evidence, although not conclusive, regarding its possible role in the death pathway and other relevant information (dependence on de novo protein synthesis, interspecies conservation, etc).

*The plus and minus symbols indicate whether the respective regulatory behavior of the gene under investigation argues for (+) or against (−) its possible role in the GC-induced cell death pathway.

†Only informative if gene is regulated in sensitive parental system (CCRF-CEM).

‡Only informative if gene is regulated in sensitive, but not resistant, parental system (CCRF-CEM).

§Only relevant if death pathway is conserved in mouse thymocytes and childhood ALL.

‖Only informative if gene is regulated in CEM-C7H2 after 6-hour GC treatment.

the first 24 to 48 hours. All children except BCP-ALL-24 scored as "prednisolone good responders" by day 8, as defined by the BFM protocol (< 1000 blasts/μL on day 8). Further details on in vivo and ex vivo treatment and purification of lymphoblasts for expression profiling are detailed below and in the Supplemental Materials' sections 1 and 2 on the *Blood* website; click on the Supplemental Materials link at the top of the online article.

In vitro models of GC sensitivity, resistance, and restored sensitivity. As in vitro models for GC-induced leukemia apoptosis we used CCRF-CEM-C7H2 T-ALL cells[28] and preB697 BCP-ALL cells.[29] Both cell lines undergo almost complete cell death after 48- to 72-hour incubation with 10^{-7} M dexamethasone. As GC resistance models, CEM-C1,[30] CEM-C7R1,[31] CEM-C7R1^{low}, and PreB697-R4G4 (described in the supplement, section 1.2) were used. GC sensitivity was restored in resistant CEM-C1 cells by stable, constitutive expression of rat GR^{wt} (CEM-C1^{ratGR} clone C1-4G4),[30] and in resistant CEM-C7R1 by high-level expression of human GR^{A458T} (CEM-C7R1$^{dim\text{-}high}$) (Supplemental Materials section 2.2).

Cycloheximide treatment. To assess whether gene regulations were dependent upon de novo protein biosynthesis, we used CCRF-CEM-C7H2 cells treated with dexamethasone in the presence of cycloheximide (CHX) for 6 hours.

Mouse models. For in vitro GC response, thymocytes from 4- to 6-week-old CD-1 mice were treated with 10^{-7} M dexamethasone or 0.1% ethanol as vehicle control for 4 hours. To determine the in vivo response to GCs, 4- to 6-week-old CD-1 mice were injected intraperitoneally with 0.2 mg dexamethasone per mouse or phosphate-buffered saline as control, and their thymocytes were used for RNA preparation.

Healthy donors. After giving written informed consent, 2 healthy adults were treated with dexamethasone according to a similar protocol as that used for the children. Subsequently, their peripheral blood mononuclear cells were purified by Lymphoprep separation (AXIS-Shield, Rodelokka, Norway) and used for RNA preparation.

Purification of peripheral lymphoblasts from patients

Mononuclear cells were purified from peripheral EDTA blood by centrifugation on Lymphoprep and the percentages of blasts determined by fluorescence-activated cell-sorting (FACS) analysis. If blast purity was less than 90%, the blasts were enriched to 90% or more by magnetic field separation as detailed in the supplement, section 2.1.

GC bioactivity assay

GC bioactivity (GBA) in the patients' plasma prior to and during GC therapy was measured from 20-μL samples (cell supernatants after Lymphoprep separation) using a recombinant cell bioassay in which COS-1 cells are transfected with expression vectors encoding human GR and a nuclear receptor coregulator, ARIP3, together with an appropriate reporter gene.[32]

Generation and characterization of transfected cell lines

The production of CEM-C1^{ratGR}-4G4 has been described.[30] CEM-C7R1$^{dim\text{-}high}$ and CEM-C7R1$^{dim\text{-}low}$ were generated by stable transduction of GC-resistant CEM-C7R1 cells (which contained 2 mutated GR alleles) with a retroviral vector expressing human GR containing the point mutation A458T. CEM-C7R1$^{dim\text{-}high}$ showed high-level GR expression and was sensitive to GC-induced apoptosis, whereas CEM-C7R1$^{dim\text{-}low}$ expressed much lower levels and remained resistant (Figure S1).

Microarray analysis and quality parameters

For microarray analysis, 1.5 μg high-quality total RNA (Supplemental Materials section 2) was processed into a biotinylated hybridization target using corresponding kits from Affymetrix, hybridized to U133 Plus 2.0 microarrays and analyzed in an Affymetrix scanner 3000. Image analysis was performed with the Affymatrix GCOS software (Santa Clara, CA). Data processing and analysis was performed in "R" (Bioconductor, http://www.bioconductor.org) using the robust multiarray analysis (RMA) method for normalization and Bioconductor's hgu133plus2 annotation package for annotation. Normalized expression values (E values) were inserted into a database using Bioconductor's developmental package maDB and used to calculate regulation values (M values). Section 4 in the Supplemental Materials summarizes quality parameters, including 3′ to 5′ signal ratios and percentages of "present calls" for each array, variance in technical replicates, regulation of methotrexate (MTX) response genes, and real time reverse transcriptase–polymerase chain reaction (RT-PCR) verification results for 25 genes.

Data verification by real time RT-PCR

Total RNA (500 ng) was reversely transcribed into cDNA using Superscript II (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. cDNA (100 ng) was assayed on microfluidic cards containing 24 human genes in duplicate (Applied Biosystems, Foster City, CA). For *MYC, SNF1LK, BTNL9, GZMA,* and *GPR65/TDAG-8,* individual premanufactured ABI assays were used (Supplemental Materials section 4.3).

Table 2. Biological systems: patients

Sample ID	Sex	Age, y	Risk*	Molecular diagnosis†	MRD‡	WBC g/L	Immunophenotype§	Clustering‖	GBA¶		
									0 h	6 h	24 h
T-ALL-2	M	8.5	SR	NAD	LR	30.9	CD3, 4, 5, 8, 10	T-ALL	62.5	189.6	179.8
T-ALL-20	M	5	MR	NAD	IR	135.6	CD2, 3, 5, 7	T-ALL	48.7	110.7	139.0
T-ALL-25	F	10.3	MR	t(8:14)(q24:11)	IR	66.8	CD2, 3, 5, 7, 8	T-ALL	78.4	85.8	197.2
B-ALL-13	M	5.9	SR	t(8:14)(q24:11)	LR	13.4	CD10, 13, 19, 34	Not assigned	62.3	66.4	77.6
B-ALL-17	F	14.7	DBA	Hyperploidy >50	DBD	44	CD10, 13, 19, 33, 34	Hyperploidy	37.1	94.9	132.5
B-ALL-24	F	2.6	HR	Low hyperploidy	IR	8.3	CD10, 19, 34	Not assigned	50.9	64.6	89.6
B-ALL-31	F	17.2	MR	Hyperploidy >50	IR	8.6	CD10, 19, 34	Hyperploidy	64.9	88.8	114.6
B-ALL-32	F	3.7	MR	t(4:8), TEL/AML	IR	26.4	CD19	TEL-AML	54.4	114.2	100.7
B-ALL-33	M	2.5	MR	Low hyperploidy	IR	79.3	CD10, 19, 34	Hyperploidy	38.2	151.2	65.2
B-ALL-37	F	15.1	MR	Low hyperploidy	IR	4.1	CD10, 19, HL-DR	Not assigned	69.5	77.1	124.3
B-ALL-38	M	3.2	MR	TEL/AML	IR	5	CD10, 19	TEL-AML	43.2	119.4	92.4
B-ALL-40	M	17.3	HR	NAD	HR	82.5	CD10, 19, 34	Not assigned	76.6	130.9	131.3
B-ALL-43	F	1.6	SR	NAD	ND	53.1	CD10, 19	TEL-AML	50.9	71.7	116.1
Adult-BCP	M	72.4	NA	ND	NA	16.2	CD19; IgG3#	ND	86.9	120.0	112.9

SR indicates standard risk; NAD, nothing abnormal detected in the above assays; LR, low risk; MR, medium risk; IR, intermediate risk; DBA, died before risk assignment; HR, high risk; ND, not done; and NA, not applicable.

*Risk group assignment according to the BFM 2000 protocol (BFM-ALL Study Group): SR indicates standard risk (prednisolone good response, as well as complete cytomorphologic bone marrow remission on day 33, and neither BCR/ABL nor MLL/AF4, and MRD-negative on day 33); MR, medium risk (as standard but MRD-positive on day 33); HR, high risk (prednisolone poor response or Bcr/Ab1 or MLL/AF4 or MRD-positive on day 77).

†Molecular diagnosis included ploidy determination by standard karyotyping, detection of BCR/ABL, BCR/ABL1, E2A/PBX1, MLL-1/AF-4, TEL/AML, SIL/TAL translocations by PCR and reconfirmation by in situ hybridization.

‡Risk assignment according to MRD (minimal residual disease) detection by T-cell receptor or immunoglobulin rearrangement-specific PCR.

§CD marker expression in 80% or more of blasts as determined by direct immunofluorescence and FACS analysis.

‖Entity assignment by gene clustering according to Ross et al[33] (Figure S2).

¶GC bioactivity in nM cortisol equivalents: means of 2 measurements at 0, 6, and 24 hours.

#Data derived from expression profiling only.

Results

To identify possible common upstream component(s) of the cell death pathway induced by GCs in childhood ALL, we exploited a comparative expression profiling strategy (Figure 1, Table 1) using "whole-genome" arrays (Affymetrix; U133 plus 2.0) and a number of additional biological systems in which GCs do or do not induce apoptosis (Tables 2-3). To this end, we first determined the expression profiles of peripheral lymphoblasts from 13 children with ALL prior to and under treatment with GCs for 6 to 8 hours and 24 hours (for GC bioactivity levels in the sera and other clinical features, see Tables 2-3). The expression profiles of these 39 arrays were used to generate 26 comparisons (0 hours versus 6 to 8 hours, and 0 hours versus 24 hours) that were entered into a database (GC response—childhood ALL database in Figure 1).

Regulation of previously identified candidates

First, we used this database to investigate whether genes implicated in GC-induced apoptosis in experimental systems (reviewed in Schmidt et al[4]) might be regulated in children with ALL. As shown in Table 4, of the current candidate genes, LDH-A/lactate dehydrogenase-A,[24] GPR65/TDAG-8,[21] MAP2K3/MAP kinase kinase 3,[27] GZMA/granzyme A,[20] MYC/c-myc,[23] NR3C1/GR,[24] and BCL2L11/Bim,[8,19,36] none was regulated more than 2-fold in most children, as might be expected from key players in a conserved pathway. Two deserve further attention: the GR that was induced in all 3 T-ALLs, and the proapoptotic BH3-only molecule Bim where probe sets corresponding to this locus, but not necessarily to the known major bim transcripts, were induced in up to 6 of 13 children ("Discussion"). We further investigated the remaining 26 genes in a currently established list of experimental system–derived candidates[4] but, with the exception of FKBP51, SOCS1, and DDIT4/Dig2, which will be discussed further, none scored in more than 4 children (Table S1).

Genes frequently regulated in childhood ALL

To directly define candidate genes relevant for induction of apoptosis by GC in childhood ALL, we first identified all probe sets that revealed M values of 1 or more (2-fold regulation) in at least 7

Table 3. Biological systems: additional systems

System	File name prefix	Sample ID	Exposure
Healthy PBLs	HD-	STS-1, RPK-2	In vivo
Sensitive primary cells	IV-	BCP-ALL-40	Ex vivo
Sensitive cell lines*	S-line-	C7H2, Pre B 697	In vitro
Resistant cell lines†	R-line-	C7R1, CEM-C1, Pre B-R4G4, C7R1$^{-dim-high}$	In vitro
Converted cell lines‡	C-line-	C7R1$^{-dim-low}$, CEM-C1^{ratGR}	In vitro
Cycloheximide sensitivity	CHX-	CEM-C7H2	In vitro
Mouse thymocytes	Mouse-	CD1	In vivo
Mouse thymocytes	Mouse-	CD1	Ex vivo

*The GC-sensitive human ALL cell lines CCRF-CEM-C7H2[28] and PreB-697.[29]

†GC-resistant cell lines CEM-C7R1[31] and CEM-C1[30,34] have been published.

‡"Converted" refers to cell lines in which GC sensitivity was restored either by stable transfection with wild-type rat GR (CEM-C1^{ratGR}) or by high-level expression of human GR carrying the GR^{dim} mutation[35] stably transfected into C7R1 (C7R1$^{dim-high}$). C7R1$^{dim-low}$ expressed less GR^{dim} and remained GC-resistant (Figure S1).

Table 4. Regulation of top candidate genes derived from experimental systems

	T-ALL (3)			BCP-ALL (10)		
Symbol	Up	Down	Both	Up	Down	Both
LDH A	0	0	0	0	0	0
GPR65	0	0	0	0	0	0
MAP2K3	0	0	0	1	0	0
GZMA	0	1	0	1	2	1
MYC	1	0	0	1	3	0
NR3C1	3	0	0	2	0	0
BCL2L11	2	0	0	4	0	0

The data summarize the number of children where the indicated gene was found to be up-regulated (Up), down-regulated (Down), or regulated in different direction at the two time points investigated (Both). In all instances the cut-off was an M value of 1 or more or −1 or less. The complete data set for these and additional 26 genes found to be frequently regulated in experimental systems[4] is shown in Table S1.

of 13 patients (128 probe sets, 104 genes; Table S2). Since we were mainly interested in primary response genes, we focused on the probe sets within this collection that were regulated with an M value of 0.7 or more at 6 to 8 hours in at least 6 of 13 patients. Twenty-five induced and 37 repressed probe sets ("top 62"), corresponding to 19 and 30 genes, respectively, met this requirement. Within the limitations of the assay system, this collection can be assumed to contain the critical upstream gene(s) responsible for GC-induced apoptosis, although hidden by genes unrelated to the death response. To distinguish the former from the latter, we performed comparative expression profiling using the additional biological systems shown in Tables 2-3. The expression profiles from these systems prior to and after dexamethasone exposure were used to generate a second database (GC-response genes—additional systems database 2 in Figure 1). Subsequently, we determined the performance of the "top 62" probe sets derived from the children with ALL in this database (Table S4).

A cluster of regulated cell-cycle genes

Thirty-four of the 37 repressed probe sets resulted in a remarkably distinct pattern: they were highly expressed in cell lines in contrast to all other systems (Table S5). They remained unregulated in 6 of 7 cell lines, in peripheral lymphocytes from healthy controls and in mouse thymocytes, but were repressed in the adult patient, resembling the situation in children. Moreover, in patient BCP-ALL-40, the 34 probe sets were strongly regulated in vivo but much less so during ex vivo treatment, whereas the opposite behavior was shown by known GC response genes like *FKBP51* (Tables S3 and

Table 5. Comparative expression profiling defined candidate genes for GC-induced apoptosis

		Childhood ALL					Additional systems							
			Frequency in children†						In vitro response‖				Mouse¶	
Symbol	Description	Mean ± SD*	PA	6 h	24 h	Opp	Adult‡	HD§	Sens	Res	Conv	CHX#	In vivo	In vitro
PFKFB2	PFK2.2	1.6±0.6	11	9	10	0	Y	0	2	1	2	Y	N	N
BTNL9	Butyrophilin-like 9	1.6±0.7	11	9	8	0	N	0	2	1	1	NA	N	N
SNFILK	SNF1-like kinase	1.7±0.9	11	5	10	2	Y	0	2	1	NA	NA	Y	N
FKBP5	FK506 binding protein 51	1.8±0.6	10	7	9	0	Y	2	3	3	2	Y	Y	Y
ZBTB16?	ZFand BTB domain 16?	2.6±1.6	10	7	8	0	Y	2	2	1	2	NA	N	N
KIF26A	Kinesin family member 26A	1.6±0.3	10	7	6	0	N	0	1	0	NA	NA	N	N
SLA	Src-like-adaptor	1.7±0.5	9	7	9	0	Y	0	3	2	Y/N	N	N	N
SOCS1	SOCS-1	2.5±1.0	9	6	8	0	Y	2	2	1	Y/N	N	Opp	Opp
DDIT4	DNA-damage-ind.transcript 4	1.9±0.7	9	6	8	0	Y	2	3	2	2	Y	Y	Y
GBP4	Guanylate binding protein 4	−1.4±0.2	9	5	8	0	N	0	1	0	NA	NA	N	N
MGC17330	HGFL gene	1.3±0.3	9	5	9	0	Y	1	3	1	2	Y	Y	Y
ZFP36L2	Zinc finger protein 36	1.4±0.5	8	5	7	0	Y	0	3	2	Y/N	Y	Y	Y
Unknown	Unknown	1.5±0.3	8	5	8	0	N	0	2	0	2	NA	NA	N/A
EPPK1	Epiplakin 1	1.9±0.6	7	7	6	0	N	0	1	0	NA	NA	Y	N
P2RY14	Purinergic receptor P2Y	1.7±0.7	7	5	6	0	N	0	2	1	0	NA	Y	Y
FGR	Gardner-Rasheed v-fgr	1.5±0.3	7	5	4	0	N	0	1	0	NA	NA	N	N
WFS1	Wolframin	1.7±0.3	7	5	6	0	N	0	3	1	Y/N	N	N	N
ARPP-21	cAMP-regulated PP21	−1.6±0.7	7	5	3	0	N	0	0	0	NA	NA	N	Y
SERPINA1	Proteinase inhibitor, clade A	1.8±0.5	7	6	4	3	N	0	0	0	NA	NA	N	Opp
GIMAP7	GTPase, IMAP family M7	−1.3±0.3	7	6	3	3	Y	0	0	1	NA	NA	N	N
MYCPBP	c-myc promoter BP	1.7±0.1	7	5	3	2	N	0	0	0	NA	NA	N	N
LGALS3	Galectin 3	1.3±0.4	7	5	3	1	N	0	0	0	NA	NA	N	N

PA indicates patients analyzed; Opp, number of patients showing regulation in the opposite direction; HD, healthy donor; Sens, GC-sensitive systems; Res, GC-resistant systems; Conv, converted systems; and NA, not applicable.

*Mean ± SD of M values from regulated samples after 6 to 8 hours of GC exposure in vivo.

†Number of patients with M values of 1.0 or higher after 6 hours, 24 hours, and 6 or 24 hours in vivo exposure to GCs (for treatment details see Supplement).

‡Regulation (Y), or lack thereof (N), in lymphoblasts from an adult patient with ALL treated with GCs in vivo.

§2, 1, or 0 indicates whether the peripheral blood lymphocytes of both (2), either 1 (1), or none (0) of the 2 healthy volunteers responded with M values of 1.0 or higher after 6-hour and/or 24-hour in vivo exposure to GCs (for treatment details see Supplement).

‖For each gene, the number of in vitro systems showing M values of 1.0 or higher after 6-hour and/or 24-hour exposure to 10^{-7} M dexamethasone is indicated. GC-sensitive systems: BCP-ALL-40 (treated ex vivo), CEM-C7H2, PreB-697; GC-resistant systems: CEM-C1, CEM-C7R1, CEM-C7R1$^{dim\text{-}low}$, PreB-697-R4G4; converted systems: CEM-C1^{ratGR}, CEM-C7R1$^{dim\text{-}high}$. If NA, the gene was not regulated in CEM-C7H2 (and hence cannot be "converted"); Y/N indicates regulated in C1^{ratGR} but not in CEM-C7R1$^{dim\text{-}high}$.

¶Y and N indicate whether (Y) or not (N) the respective gene was regulated in CD1 mouse thymocytes after 4-hour exposure to dexamethasone in vivo and/or in vitro, as indicated.

#Genes regulated (M ≥ 1.0) in CEM-C7H2 cells after 6-hour exposure to 10^{-7} M dexamethasone in the presence of 10 μg/mL cycloheximide (CHX) are indicated with Y; those no longer regulated in the presence of CHX with N. NA denotes genes that were not regulated by GC in CEM-C7H2 cells in the absence of CHX.

S4 for regulation of these probe sets in children and additional systems, respectively). Combined with the fact that none of the 27 genes corresponding to these 34 probe sets has previously been reported to be GC regulated, the above results suggested that they are not direct transcriptional GC targets ("Discussion"). Since all of them are involved in late cell-cycle progression,[37,38] we referred to this coordinately regulated group as "cell-cycle genes." Because the aim of this study was to identify primary response genes in the GC-induced cell death pathway (which these genes are probably not), and since previous observations suggested that cell-cycle arrest is not required for cell death,[39] we focused our further analyses on the remaining 28 probe sets.

Candidate genes for GC-induced apoptosis

After reduction of the 28 probes sets to their corresponding genes (by using the M values of the probe sets with the strongest regulation), the performance of the resulting 22 candidate genes in the children and additional systems was compiled in Table5. Although not formally ruling out any gene, the combined information might prove useful for selection of candidates for future functional analyses. Thus, genes no longer regulated in the presence of the translation inhibitor cycloheximide *(SOCS1, SLA, WFS1)*, and/or genes not regulated in any of the additional in vivo, ex vivo, and in vitro systems of GC-induced apoptosis *(ARPP-21, SERPINA1, MYCBP, LGALS3)* may not be direct transcriptional GC targets. In contrast, absent or reduced gene regulation in 4 instances of in vitro GC resistance and/or in mature peripheral blood lymphocytes (which are considered to be insensitive to GC-induced cell death when in a resting state)[40,41] might argue in favor of functional relevance of the respective gene (eg, *PFKFB2, BTNL9, SNF1LK*). Finally, genes coregulated in childhood ALL and mouse thymocytes (eg, *SNF1LK, FKBP5, DDIT4*) would qualify as possible components of a canonical pathway conserved between species and systems (mouse thymocytes, human ALL cells).

In conclusion, we generated 2 databases encompassing comprehensive lists of GC-regulated candidate genes in children with ALL and a number of additional systems, permitting immediate analysis of any gene with respect to its regulation, and thus potential significance for GC-induced apoptosis. The study provided important evidence for some of the key questions in the field: several current model-based hypotheses could essentially be ruled out for childhood leukemia, and the number of potential candidates for a common upstream regulator in mouse thymocyte and human leukemia cells was dramatically reduced. Gene induction rather than gene repression might account for cell death in childhood ALL, although this conclusion must be viewed with caution since down-regulation may be more difficult to detect than gene induction, and only a handful of genes qualified for a critical upstream component of the GC-evoked death pathway in children with ALL, most notably 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 *(PFKFB2)*, a key regulator of glucose metabolism; zinc finger and BTB domain–containing gene 16 *(ZBTB16)*, a putative transcription factor, and SNF1-like kinase *(SNF1LK)*, a protein kinase implicated in cell-cycle regulation.

Discussion

Despite its clinical relevance and decades of research, the molecular basis of GC-induced leukemia apoptosis has remained a mystery. Numerous hypotheses have been proposed, but whether the corresponding gene regulations occur in a clinical setting has not been investigated. Our study addressed, for the first time, the molecular basis of GC-induced leukemia apoptosis in a clinical setting by expression profiling using the currently most complete probe collection (U133 plus 2.0; 54 000 probe sets). The underlying hypothesis was that GCs induce apoptosis by altering gene expression at the mRNA level and that the basic mechanism is shared among different children with ALL. Within these premises and the limitations of the technology (presence of an appropriate probe set on the array, regulation 2-fold or more), the key component(s) of the respective pathway should become apparent given the number of children investigated. To provide further information regarding the possible significance of the identified genes, we also analyzed a number of additional systems.

Previously identified candidates and related hypotheses

Regarding candidates and related hypotheses derived from experimental systems, our data argued against a general role of lactate dehydrogenase A,[24] granzyme A,[20] TDAG-8,[21] MAP kinase kinase 3,[27] and c-myc[23] in cell death induction in childhood ALL, although some of these genes may be relevant for cell death induction in experimental systems or in subgroups of children. In the case of c-myc, TDAG8, GZMA, bim, and GR, the findings were further reconfirmed by real-time RT-PCR for all patients where sufficient mRNA remained (section 4.3 in the Supplemental Materials). GR autoinduction, which we and others have proposed to be important for GC-induced apoptosis in the CCRF-CEM model for T-ALL,[24,42,43] was observed in all 3 patients with T-ALL, but only in 2 of 10 patients with BCP-ALL. Although the number of patients is too small to draw firm conclusions for subgroups, GR autoinduction may be relevant for patients with T-ALL, an entity that shows a relatively high rate of tumor lysis syndrome.[44] Three of the 8 probe sets for the BCL2L11/Bim locus on chromosome 2 were induced 2-fold or more in our children with ALL. Probe set 1 555 372_s_at, induced most frequently (6 of 13), matches the 3′ end of a multiple myeloma–derived cDNA referred to as Bam.[45] The reported Bam mRNA starts 94 bp upstream from the BH3-containing Bim exon and encodes a predicted 73–amino acid protein with a BH3 domain and 40 amino acids not present in any known Bim protein. The second probe set, 225 606_at, maps about 1 kb downstream from the currently known 3′ end of Bim transcripts and might have resulted from alternative polyadenylation. It was regulated in 4 of the 6 children who showed induction of probe set 1 555 372_s_at. Probe set 1 558 143_s_at recognized the reported 3′ end of all major Bim isoforms (including BimEL, BimL, and BimS)[46] and was induced in 3 of the 6 children after 24 hours, but not after 6 hours. Thus, although the complexity of the BCL2L11 locus precludes final conclusions, transcripts from this locus may contribute to cell death induction in at least a subgroup of children either as primary GC targets or as downstream effector molecules.

Three of the previously identified candidates (*FKBP5/FKBP51, DDIT-4/Dig2,* and *SOCS-1*) were reconfirmed in most patients. *FKBP51* has recently been proposed as general indicator of GC sensitivity, and a corresponding assay was developed.[47,48] It is a GR cochaperone that is transcriptionally induced by GC[48,49] and competes with *FKBP52* for dynein binding sites,[50,51] thereby reducing nuclear transport of the ligand-bound receptor. Its induction reduces transcriptional GC effects, and cells overexpressing *FKBP51* are more resistant to GC-induced apoptosis.[50] *DDIT4/Dig-2* has been suggested to mediate both prosurvival and proapoptotic functions[52] and its overexpression, like that of *FKBP51,*

reduced sensitivity to dexamethasone-induced apoptosis.[53] Provided these findings in the cell lines can be extended to patients, these 2 genes, although regulated by GCs in many systems, may not be causally involved in cell death induction. SOCS1, a potentially antisurvival protein, as implicated by its name ("suppressor of cytokine signaling"), was induced in 9 of 13 patients, but its regulation in CCRF-CEM cells was sensitive to cycloheximide and its expression repressed in mouse thymocytes. However, should the data in the model systems be irrelevant for the clinical situation, SOCS1, by virtue of its function as an antisurvival protein, remains 1 of the most attractive candidates.

Cell cycle genes: an example of apparent "gene regulations" caused by population shift?

The significant decrease in expression levels of the cell-cycle genes (a set of genes known to be expressed in the G_2 and/or M phases of the cell cycle) observed after GC exposure in 11 of 13 children and the adult subject deserves further discussion. Based on the arguments put forward in "Results," we consider it unlikely that these genes are direct transcriptional GC targets. A possible explanation for the decline in expression levels after GC treatment might be that proliferating cells within the tumor were retained in the bone marrow, migrated out of the bloodstream, and/or were selectively killed by GCs. Thus, the observed changes in gene expression might reflect a treatment-induced shift from a more proliferative population of leukemia cells at 0 hours toward a less proliferative population after 6 and 24 hours rather than resulting from direct transcriptional regulation by GCs. Since in vitro migration does not occur and apoptotic cells are not effectively removed, this phenomenon might be more easily detectable in vivo than in tissue culture.

Candidates conserved between human leukemia cells and mouse thymocytes

Whether GC-induced apoptosis in mouse thymocytes and human patients with ALL is controlled by the same gene(s) is of considerable interest for various reasons, including functional testing of candidates in vivo. Our study uncovered only a small number of genes coordinately regulated in mice and most children with ALL (Table 4). With the possible exception of *SNF1LK* (which will be discussed in "New candidates"), these candidates do not appear very promising: two of them (*FKBP51* and *DDIT4*) protected cells from GC-induced apoptosis,[50,53] and none of the remaining coregulated genes has been implicated in apoptotic or survival pathways thus far, supporting the notion that GC-induced apoptosis in mouse thymocytes and human lymphoblastic leukemia cells might be controlled by different genes.

Gene induction versus gene repression

Another general suggestion from Table 4 concerns the question of whether GC-induced leukemia apoptosis results from gene induction or repression. After subtraction of the cell-cycle genes (which may not be direct GC targets nor responsible for cell death induction), only 3 down-regulated genes remained: *GBP4* (guanylate-binding protein 4),[54] *ARPP-21* (cAMP-regulated phosphoprotein 21);[55] and *GIMAP7* (GTPase immune-associated protein 7, also known as human immune-associated nucleotide, hIAN7).[56] Since these genes have not been implicated in any known death pathway and performed rather moderately in the various systems (Table 4), a prominent role as initiator of the death pathway seems unlikely. Thus, the data from the patients do not support the cell line–derived conclusion that GC-induced leukemia depends on gene repression.[57]

New candidates

A particularly interesting candidate in the upper part of Table 5 is *SNF1LK,* a member of the SNF/AMPK family of protein kinases. Although its role is currently not well understood, it has been implicated in regulation of the G_2/M phase of the cell division cycle,[58] and shows homology to genes controlling carbohydrate metabolism in plants.[59] Thus, *SNF1LK* may be involved in the observed effects on cell-cycle genes or may lead to (potentially harmful) metabolic alterations. It was induced in 11 of 13 children, the adult patient, and in mouse thymocytes in vivo*,* making it a possible candidate for a critical upstream component in a pathway conserved between species. However, the reason for its repression in 2 children with T-ALL (reconfirmed by real-time RT-PCR; Supplemental Materials section 4.3) is unclear. The functions of *KIF26A* and *BTNL-9* are currently unknown; hence, their potential role in apoptosis induction is difficult to assess. *KIF26A* belongs to the N11 kinesins, a subgroup of the large kinesin family that has been implicated in cellular transport processes.[60] *BTNL-9* shares structural similarity with butyrophilin, a structural component of the human milk fat globule.[61] This gene was regulated in all 10 patients with BCP-ALL and none of the 3 patients with T-ALL, and may thus encode a protein regulating B-cell–specific GC actions. *SLA* (Src-like adaptor) encodes an adaptor protein that negatively regulates T-cell receptor (TCR) signalling.[62] If it has a similar activity in B cells, its induction (like that of *SOCS-1*) might interfere with survival signals. However, regulation of this gene was sensitive to cycloheximide in CEM-C7H2 cells and it was not induced in C7R1^{dim4} cells, although they underwent GC-induced apoptosis (again resembling *SOCS-1*). One of the most frequently regulated probe sets was 228 854_at. It mapped about 4 kb downstream of the reported 3′ end of a putative transcription factor called ZBTB16/PLZF/ZFP-145, which is required for spermatogonial stem cell renewal[63,64] and limb and axial skeletal patterning,[65] and has been found to be rearranged in promyelocytic leukemia.[66,67] As detailed in section 4.3 of the Supplemental Materials, there was a strong correlation ($R^2 = 0.8266$) between the regulation data obtained with 228 854_at in the Affymetrix screen and the *ZBTB16* real-time RT-PCR results, strongly suggesting that this probe set recognizes an undescribed variant *ZBTB16* mRNA generated by alternative polyadenylation. Thus, even though *ZBTB16* was regulated in peripheral blood lymphocytes from both healthy donors (who are supposedly relatively resistant to GC-induced apoptosis[40,41]), it remains a valid candidate for an upstream regulator of GC-induced apoptosis.

One of the most promising candidates is 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 *(PFKFB2)*, a key enzyme in glucose metabolism.[68] This gene was most frequently regulated at both the early and late time points. Its regulation was resistant to the translation inhibitor cycloheximide. Although not regulated in preB-697 cells, it was rapidly and strongly induced in CCRF-CEM-C7H2 but not, or much less so, in its GC-resistant derivatives, and showed clear induction in both "converted" models where GC sensitivity was restored by transgenesis. The gene was not regulated in peripheral blood lymphocytes from 2 healthy donors or in mouse thymocytes. Regarding possible functional consequences, recent data suggest that cellular metabolism and apoptosis might be intertwined with connections between regulation of cellular bioenergetics and apoptosis.[69,70] Malignant cells, known for their altered

glucose metabolism,[71,72] might be particularly sensitive to disturbances in glycolytic pathways. In support of this concept, regulation of glucose metabolism in thymocytes has been reported many years ago[73] and combination with 2-deoxy glucose (2-DG), a specific inhibitor of hexokinase (the enzyme phosphorylating glucose, thereby making it a substrate for further metabolic transformation), dramatically sensitized CCRF-CEM cells to cell death triggered by GCs, but not several other apoptosis inducers (K. Renner, C. Seger, and R. Kofler, manuscript submitted). Clearly, the possible functional role of *PFKFB2* (and the remaining candidates in Table 4) needs to be directly assessed. Given the limitations of existing test systems, we are currently developing lentiviral transduction systems to allow functional testing in primary cells from patients.

Relation to previously defined resistance genes

Finally, we wondered whether genes previously implicated in resistance to GCs or other chemotherapeutics might be among the probe sets frequently regulated by GCs in children with ALL. Interestingly, none of 33 genes predictive for poor GC response[74] was among the top 128 probe sets depicted in Table S2. On our microarray, we identified corresponding probe sets for 45 of 54 genes that predicted molecular treatment response in childhood ALL[75] (the remaining 10 cDNAs could not be unambiguously annotated). Two of them (*CDCA1* [cell division cycle–associated 1], probe set ID: 223 381_at; and *TTK protein kinase*, probe set ID: 204 822_at), were among our collection of regulated probe sets. Finally, we analyzed 45 genes associated with cross-resistance to 4 mechanistically distinct antileukemic agents and 139 genes related to discordant resistance to vincristine and asparaginase.[76] *MELK* (204 825_at) was the only 1 of the 45 cross-resistance predictor genes, and *HGFL/MGC17330* (221 756_at) the only member of the 139 discordant resistance predicting genes found in the top 128 probe sets (Table S2).

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References

1. Ashwell JD, Lu FW, Vacchio MS. Glucocorticoids in T cell development and function. Annu Rev Immunol. 2000;18:309-345.
2. Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. N Engl J Med. 2004;350:1535-1548.
3. Dordelmann M, Reiter A, Borkhardt A, et al. Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia. Blood. 1999;94:1209-1217.
4. Schmidt S, Rainer J, Ploner C, et al. Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance. Cell Death Differ. 2004;11:S45-S55.
5. Distelhorst CW. Recent insights into the mechanism of glucocorticosteroid-induced apoptosis. Cell Death Differ. 2002;9:6-19.
6. Planey SL, Litwack G. Glucocorticoid-induced apoptosis in lymphocytes. Biochem Biophys Res Commun. 2000;279:307-312.
7. Cidlowski JA, King KL, Evans-Storms RB, et al. The biochemistry and molecular biology of glucocorticoid-induced apoptosis in the immune system. Recent Prog Horm Res. 1996;51:457-490.
8. Bachmann PS, Gorman R, MacKenzie KL, Lutze-Mann L, Lock RB. Dexamethasone resistance in B-cell precursor childhood acute lymphoblastic leukemia occurs downstream of ligand-induced nuclear translocation of the glucocorticoid receptor. Blood. 2005;105:2519-2526.
9. Haarman EG, Kaspers GJ, Veerman AJ. Glucocorticoid resistance in childhood leukaemia: mechanisms and modulation. Br J Haematol. 2003;120:919-929.
10. Schaaf MJ, Cidlowski JA. Molecular mechanisms of glucocorticoid action and resistance. J Steroid Biochem Mol Biol. 2002;83:37-48.
11. Kofler R, Schmidt S, Kofler A, Ausserlechner MJ. Resistance to glucocorticoid-induced apoptosis in lymphoblastic leukemia. J Endocrinol. 2003;178:19-27.
12. Bamberger CM, Schulte HM, Chrousos GP. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. Endocr Rev. 1996;17:245-261.
13. Laudet V, Gronemeyer H. The nuclear receptor facts book. London, United Kingdom: Academic Press; 2002.
14. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science. 1998;281:1322-1326.
15. Krammer PH. CD95's deadly mission in the immune system. Nature. 2000;407:789-795.
16. Nagata S. Apoptosis by death factor. Cell. 1997;88:355-365.
17. Nicholson DW. Caspase structure, proteolytic substrates, and function during apoptotic cell death. Cell Death Differ. 1999;6:1028-1042.
18. Shi Y. Caspase activation: revisiting the induced proximity model. Cell. 2004;117:855-858.
19. Wang Z, Malone MH, He H, McColl KS, Distelhorst CW. Microarray analysis uncovers the induction of the pro-apoptotic BH3-only protein Bim in multiple models of glucocorticoid induced apoptosis. J Biol Chem. 2003;278:23861-23867.
20. Yamada M, Hirasawa A, Shiojima S, Tsujimoto G. Granzyme A mediates glucocorticoid-induced apoptosis in leukemia cells. FASEB J. 2003;17:1712-1714.
21. Malone MH, Wang Z, Distelhorst CW. The glucocorticoid-induced gene tdag8 encodes a pro-apoptotic G protein-coupled receptor whose activation promotes glucocorticoid-induced apoptosis. J Biol Chem. 2004;279:52850-52859.
22. Löffler M, Tonko M, Hartmann BL, et al. c-myc does not prevent glucocorticoid-induced apoptosis of human leukemic lymphoblasts. Oncogene. 1999;18:4626-4631.
23. Medh RD, Wang A, Zhou F, Thompson EB. Constitutive expression of ectopic c-Myc delays glucocorticoid-evoked apoptosis of human leukemic CEM-C7 cells. Oncogene. 2001;20:4629-4639.
24. Tonko M, Ausserlechner MJ, Bernhard D, Helmberg A, Kofler R. Gene expression profiles of proliferating versus G1/G0 arrested human leukemia cells suggest a mechanism for glucocorticoid-induced apoptosis. FASEB J. 2001;15:693-699.
25. Obexer P, Certa U, Kofler R, Helmberg A. Expression profiling of glucocorticoid-treated T-ALL cell lines: rapid repression of multiple genes involved in RNA-, protein- and nucleotide synthesis. Oncogene. 2001;20:4324-4336.
26. Kofler R. The molecular basis of glucocorticoid-induced apoptosis of lymphoblastic leukemia cells. Histochem Cell Biol. 2000;114:1-7.
27. Miller AL, Webb MS, Copik AJ, et al. p38 MAP kinase is a key mediator in glucocorticoid-induced apoptosis of lymphoid cells: correlation between p38 MAPK activation and site-specific phosphorylation of the human glucocorticoid receptor at serine 211. Mol Endocrinol. 2005;19:1569-1583.
28. Strasser-Wozak EMC, Hattmannstorfer R, Hála M, et al. Splice site mutation in the glucocorticoid receptor gene causes resistance to glucocorticoid-induced apoptosis in a human acute leukemic cell line. Cancer Res. 1995;55:348-353.
29. Findley HW Jr, Cooper MD, Kim TH, Alvarado C, Ragab AH. Two new acute lymphoblastic leukemia cell lines with early B-cell phenotypes. Blood. 1982;60:1305-1309.
30. Geley S, Hartmann BL, Hala M, et al. Resistance to glucocorticoid-induced apoptosis in human T-cell acute lymphoblastic leukemia CEM-C1 cells is due to insufficient glucocorticoid receptor expression. Cancer Res. 1996;56:5033-5038.
31. Hala M, Hartmann BL, Böck G, Geley S, Kofler R. Glucocorticoid receptor gene defects and resistance to glucocorticoid-induced apoptosis in human leukemic cell lines. Int J Cancer. 1996;68:663-668.
32. Raivio T, Palvimo JJ, Kannisto S, Voutilainen R, Janne OA. Transactivation assay for determination of glucocorticoid bioactivity in human serum. J Clin Endocrinol Metab. 2002;87:3740-3744.
33. Ross ME, Zhou X, Song G, et al. Classification of pediatric acute lymphoblastic leukemia by gene expression profiling. Blood. 2003;102:2951-2959.
34. Zawydiwski R, Harmon JM, Thompson EB. Glucocorticoid-resistant human acute lymphoblastic leukemic cell line with functional receptor. Cancer Res. 1983;43:3865-3873.
35. Reichardt HM, Kaestner KH, Tuckermann J et al. DNA binding of the glucocorticoid receptor is not essential for survival. Cell. 1998;93:531-541.
36. Abrams MT, Robertson NM, Yoon K, Wickstrom E. Inhibition of glucocorticoid-induced apoptosis by targeting the major splice variants of BIM with siRNA and shRNA. J Biol Chem. 2004;279:55809-55817.
37. Whitfield ML, Sherlock G, Saldanha AJ, et al. Identification of genes periodically expressed in the human cell cycle and their expression in tumors. Mol Biol Cell. 2002;13:1977-2000.

38. Fontijn RD, Goud B, Echard A, et al. The human kinesin-like protein RB6K is under tight cell cycle control and is essential for cytokinesis. Mol Cell Biol. 2001;21:2944-2955.
39. Ausserlechner MJ, Obexer P, Böck G, Geley S, Kofler R. Cyclin D3 and c-myc control glucocorticoid-induced cell cycle arrest but not apoptosis in lymphoblastic leukemia cells. Cell Death Differ. 2004;11:165-174.
40. Nieto MA, González A, Gambón F, Díaz-Espada F, López-Rivas A. Apoptosis in human thymocytes after treatment with glucocorticoids. Clin Exp Immunol. 1992;88:341-344.
41. Brunetti M, Martelli N, Colasante A, et al. Spontaneous and glucocorticoid-induced apoptosis in human mature T lymphocytes. Blood. 1995;86: 4199-4205.
42. Ramdas J, Liu W, Harmon JM. Glucocorticoid-induced cell death requires autoinduction of glucocorticoid receptor expression in human leukemic T cells. Cancer Res. 1999;59:1378-1385.
43. Riml S, Schmidt S, Ausserlechner MJ, Geley S, Kofler R. Glucocorticoid receptor heterozygosity combined with lack of receptor auto-induction causes glucocorticoid resistance in Jurkat acute lymphoblastic leukemia cells. Cell Death Differ. 2004;11:S65-S72.
44. Tiley C, Grimwade D, Findlay M, et al. Tumour lysis following hydrocortisone prior to a blood product transfusion in T-cell acute lymphoblastic leukaemia. Leuk Lymphoma. 1992;8:143-146.
45. Claudio JO, Masih-Khan E, Tang H et al. A molecular compendium of genes expressed in multiple myeloma. Blood. 2002;100:2175-2186.
46. U M, Miyashita T, Shikama Y, Tadokoro K, Yamada M. Molecular cloning and characterization of six novel isoforms of human Bim, a member of the proapoptotic Bcl-2 family. FEBS Lett. 2001;509:135-141.
47. Vermeer H, Hendriks-Stegeman BI, van Suylekom D, et al. An in vitro bioassay to determine individual sensitivity to glucocorticoids: induction of FKBP51 mRNA in peripheral blood mononuclear cells. Mol Cell Endocrinol. 2004;218:49-55.
48. Vermeer H, Hendriks-Stegeman BI, van der Burg B, Buul-Offers SC, Jansen M. Glucocorticoid-induced increase in lymphocytic FKBP51 messenger ribonucleic acid expression: a potential marker for glucocorticoid sensitivity, potency, and bioavailability. J Clin Endocrinol Metab. 2003;88: 277-284.
49. U M, Shen L, Oshida T, et al. Identification of novel direct transcriptional targets of glucocorticoid receptor. Leukemia. 2004;18:1850-1856.
50. Wochnik GM, Ruegg J, Abel GA, et al. FKBP51 and FKBP52 differentially regulate dynein interaction and nuclear translocation of the glucocorticoid receptor in mammalian cells. J Biol Chem. 2004;280:4609-4616.
51. Davies TH, Ning YM, Sanchez ER. A new first step in activation of steroid receptors: hormone-induced switching of FKBP51 and FKBP52 immunophilins. J Biol Chem. 2002;277:4597-4600.
52. Shoshani T, Faerman A, Mett I, et al. Identification of a novel hypoxia-inducible factor 1-responsive gene, RTP801, involved in apoptosis. Mol Cell Biol. 2002;22:2283-2293.
53. Wang Z, Malone MH, Thomenius MJ, et al. Dexamethasone-induced gene 2 (dig2) is a novel pro-survival stress gene induced rapidly by diverse apoptotic signals. J Biol Chem. 2003;278:27053-27058.
54. Ota T, Suzuki Y, Nishikawa T, et al. Complete sequencing and characterization of 21,243 full-length human cDNAs. Nat Genet. 2004;36:40-45.
55. Brene S, Lindefors N, Ehrlich M, et al. Expression of mRNAs encoding ARPP-16/19, ARPP-21, and DARPP-32 in human brain tissue. J Neurosci. 1994;14:985-998.
56. Cambot M, Aresta S, Kahn-Perles B, de Gunzburg J, Romeo PH. Human immune associated nucleotide 1: a member of a new guanosine triphosphatase family expressed in resting T and B cells. Blood. 2002;99:3293-3301.
57. Helmberg A, Auphan N, Caelles C, Karin M. Glucocorticoid-induced apoptosis of human leukemic cells is caused by the repressive function of the glucocorticoid receptor. EMBO J. 1995;14:452-460.
58. Stephenson A, Huang GY, Nguyen NT, et al. snf1lk encodes a protein kinase that may function in cell cycle regulation. Genomics. 2004;83:1105-1115.
59. Pien S, Wyrzykowska J, Fleming AJ. Novel marker genes for early leaf development indicate spatial regulation of carbohydrate metabolism within the apical meristem. Plant J. 2001;25:663-674.
60. Miki H, Setou M, Kaneshiro K, Hirokawa N. All kinesin superfamily protein, KIF, genes in mouse and human. Proc Natl Acad Sci U S A. 2001;98: 7004-7011.
61. Peterson JA, Scallan CD, Ceriani RL, Hamosh M. Structural and functional aspects of three major glycoproteins of the human milk fat globule membrane. Adv Exp Med Biol. 2001;501:179-187.
62. Sosinowski T, Pandey A, Dixit VM, Weiss A. Src-like adaptor protein (SLAP) is a negative regulator of T cell receptor signaling. J Exp Med. 2000; 191:463-474.
63. Costoya JA, Hobbs RM, Barna M, et al. Essential role of Plzf in maintenance of spermatogonial stem cells. Nat Genet. 2004;36:653-659.
64. Buaas FW, Kirsh AL, Sharma M, et al. Plzf is required in adult male germ cells for stem cell self-renewal. Nat Genet. 2004;36:647-652.
65. Barna M, Hawe N, Niswander L, Pandolfi PP. Plzf regulates limb and axial skeletal patterning. Nat Genet. 2000;25:166-172.
66. He L, Bhaumik M, Tribioli C, et al. Two critical hits for promyelocytic leukemia. Mol Cell. 2000;6: 1131-1141.
67. Chen SJ, Zelent A, Tong JH, et al. Rearrangements of the retinoic acid receptor alpha and promyelocytic leukemia zinc finger genes resulting from t(11;17)(q23;q21) in a patient with acute promyelocytic leukemia. J Clin Invest. 1993;91:2260-2267.
68. Okar DA, Manzano A, Navarro-Sabate A, et al. PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose-2,6-bisphosphate. Trends Biochem Sci. 2001;26:30-35.
69. Hammerman PS, Fox CJ, Thompson CB. Beginnings of a signal-transduction pathway for bioenergetic control of cell survival. Trends Biochem Sci. 2004;29:586-592.
70. Plas DR, Rathmell JC, Thompson CB. Homeostatic control of lymphocyte survival: potential origins and implications. Nat Immunol. 2002;3:515-521.
71. Warburg O. Ist die aerobe Glykolyse spezifisch für die Tumoren? [Is anaerobic glycolysis specific of tumors?] Biochem Z. 1929;204:482-487.
72. Dang CV, Semenza GL. Oncogenic alterations of metabolism. Trends Biochem Sci. 1999;24:68-72.
73. Munck A. Metabolic site and time course of cortisol action on glucose uptake, lactic acid output, and glucose 6-phosphate levels of rat thymus cells in vitro. J Biol Chem. 1968;243:1039-1042.
74. Holleman A, Cheok MH, den Boer ML, et al. Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. N Engl J Med. 2004;351:533-542.
75. Cario G, Stanulla M, Fine BM, et al. Distinct gene expression profiles determine molecular treatment response in childhood acute lymphoblastic leukemia. Blood. 2005;105:821-826.
76. Lugthart S, Cheok MH, den Boer ML, et al. Identification of genes associated with chemotherapy crossresistance and treatment response in childhood acute lymphoblastic leukemia. Cancer Cell. 2005;7:375-386.

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Glucocorticoid-induced apoptosis and glucocorticoid resistance in acute lymphoblastic leukemia☆

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Abstract

Glucocorticoids (GC) induce cell cycle arrest and apoptosis in lymphoid cells, and therefore constitute a central component in the treatment of lymphoid malignancies, particularly childhood acute lymphoblastic leukemia (ALL). In spite of its clinical significance and considerable efforts in many laboratories, however, the molecular basis of GC-induced apoptosis and the clinically important resistance phenomenon remains poorly defined. The anti-leukemic GC effects are critically dependent upon sufficient expression of the GC receptor (GR) throughout the response. In ALL cell lines, this is associated with, and may depend upon, GR autoinduction. In corresponding in vitro models, GC resistance frequently results from mutations in the GR gene and/or deficient regulation of its expression. The downstream components of the pathway, i.e., the GC-regulated genes responsible for cell death induction, have been studied by microarray-based comparative expression profiling, resulting in identification of a considerable number of GC-regulated candidate genes. Their possible function in the death response is, however, still undefined. One model predicts direct regulation of the apoptotic machinery, e.g., components of the "Bcl-2 rheostat", while a complementary hypothesis suggests deleterious GC effects on essential cellular functions, such as metabolism, production of and/or response to oxygen radicals, general transcription/translation, pH and volume control, etc. These regulatory effects may entail cell death, particularly if maintained for sufficient time through GR autoinduction. The latter form of cell death may occur even in the absence of functional apoptotic machinery (e.g., when caspases are blocked), but in this case appears to entail a more necrotic morphology. Taken together, GC may induce different types of cell death through distinct molecular pathways, depending on the cellular context. GC resistance might frequently result from defective GR expression, perhaps the most efficient means to target multiple antileukemic pathways.

Keywords: Acute lymphoblastic leukemia; Glucocorticoids; GC receptor; Cell death

1. Introduction

Glucocorticoid (GC)-induced apoptosis is a phenomenon of considerable biological and clinical significance. It has been implicated in the generation of the immune repertoire and in the regulation of immune responses [1–3], and has been exploited in the therapy of lymphoid malignancies [4]. In this presentation, we discuss our current understanding of the molecular mechanism of this GC response and resistance against it, with particular emphasis on human acute lymphoblastic leukemia in vitro models (for recent reviews of this subject by other groups, see [2,5–10]). We will first summarize our previous work related to the basic apoptotic pathways used in this model, review the genes regulated by GC in various systems of GC-induced apoptosis as identified by expression profiling studies and present our current concepts regarding how GC might induce cell death. We will conclude by presenting an in vitro model for GC resistance that suggests regulatory and/or structural defects in GR gene expression as a major cause for GC resistance in leukemia cells. Our work on the molecular basis of another important anti-leukemic GC effect, i.e., GC-induced cell cycle arrest, will not be covered by this review since it has been summarized recently [11,12].

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2. Molecular mechanisms of GC-induced apoptosis

2.1. Apoptotic pathways used during GC-induced apoptosis

Two major pathways have been identified that both lead to activation of caspases the critical effector molecules in apoptosis (reviewed in [13–17]). The extrinsic pathway is initiated by activation of membrane death receptors and leads to activation of effector caspases (caspases 3, 6 and 7) via activation of inducer caspases, particularly caspase 8, in the death inducing signaling complex (DISC). The intrinsic pathway responds to intracellular signals, and leads to mitochondrial release of pro-apoptotic molecules, formation of the so-called apoptosome and activation of the above effector caspases via the initiator caspase 9. This pathway is controlled by pro- and anti-apoptotic members of the large Bcl-2 family. To address which of the two pathways is activated by GC, we have introduced the caspase 8-specific inhibitor *crmA* into CCRF-CEM human acute lymphoblastic leukemia (ALL) cells [18]. While *crmA* completely blocked apoptosis induced by antibodies to CD95/fas, it had no effect upon GC-induced apoptosis suggesting that this form of apoptosis may not critically depend on the extrinsic pathway. This conclusion is supported by studies in *crmA*-transgenic mice [19] and other human ALL cell lines [20]. In contrast, transgenic Bcl-2 expressed in our ALL cell system in a tetracycline-dependent manner delayed GC-induced apoptosis by 24 h suggesting involvement of the intrinsic pathway [21]. This notion is further supported by the observation that GC-induced apoptosis in thymocytes from APAF-1- [22,23] and caspase 9-deficient [24,25] mice is compromised (although not absent). Moreover, thymocytes from double knock-out mice lacking the BH3-only molecules Bax and Bak [26] are GC-resistant and the single "knock-outs" of the BH3-only proteins Bim [27], and Puma or Noxa [28] show partial GC resistance (Bax [29] and Bid [30] knock-outs cause mild, if any, deficiency in this response). While these and other studies defined the apoptotic pathways used during GC-induced apoptosis they did not address the question of how GC, as a regulator of gene expression, might activate these pathways.

2.2. GC-regulated genes responsible for cell death induction

GC-induced apoptosis is initiated by, and strictly dependent upon, the interaction of GC with its receptor, the GR. Upon ligand binding the GR translocates to the nucleus where it regulates the expression of a plethora of genes via a variety of molecular mechanisms (reviewed in [31]). The obvious key questions are which genes are regulated during GC-induced apoptosis, and even more importantly, which of the regulated genes are responsible for triggering cell death. Regarding the first question, an increasing number of studies have been undertaken using microarray-based expression profiling of cells undergoing GC-induced cell death [20,32–40]. We have performed a bioinformatic meta-analysis of these publications (Schmidt et al., in preparation) showing that about 900 genes have been reported as GC-regulated, but only ~70 thereof have been observed in more than one publication. No single gene was found to be regulated in all eight investigated systems (cut off: more than two-fold) and 31 genes appeared in three or more systems and/or publications (Table 1). Although only about one-third of the genome has been analyzed and technical limitations exist, this list might constitute the most informative collection of genes to date with strong evidence for GC regulation in cells prone to GC-induced apoptosis.

Regarding their function, these genes can be tentatively grouped into three classes: (i) genes directly implicated in death and survival decisions; (ii) genes whose (de)regulation might lead to cellular distress, possibly resulting in apoptotic or (apo)necrotic cell death; and (iii) genes not causal in the death response. Finally, regulation of the GR itself deserves separate mention, since its regulation determines the extent and duration of all other regulatory responses. We recently proposed that GR auto-induction might be a critical event in GC-induced apoptosis in leukemia cells [41]. In the following, we briefly discuss how GR auto-induction and other regulatory events revealed by the above expression profiling and other studies might be incorporated into a hypothesis (Fig. 1) that can now be tested experimentally by conditional gene overexpression and RNAi-mediated gene knock-down.

2.3. A two-component model for GC-induced apoptosis

The concept put forward in Fig. 1 consists of two complementary components. In the first, GC might directly activate

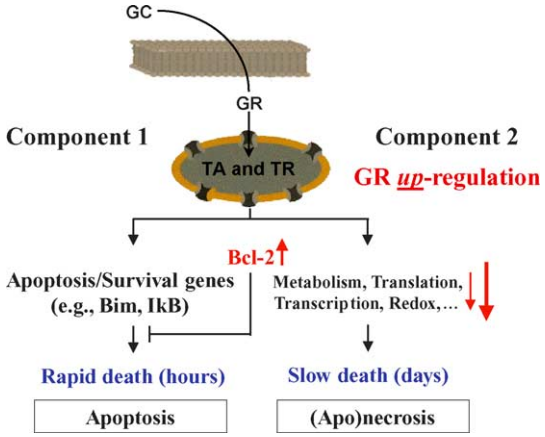

Fig. 1. Proposed model for GC-induced apoptosis GC may induce apoptosis by directly regulating typical apoptosis or survival genes, such as Bim or IkB (component 1). In the presence of overexpression of anti-apoptotic genes (such as Bcl-2), this mechanism may be blocked. Under these circumstances, a second scenario may become apparent that is based upon GC regulation of a number of vital cellular functions such as metabolism, transcription/translation, redox-, pH- and volume-control. If cellular distress is perpetuated by GR auto-induction, it may lead to (apo)necrotic cell death (component 2). For further details see text.

Table 1
Genes regulated by GC in cells prone to GC-induced apoptosis[a]

Identifier[b]	Description[c]	Reg[d]	Human[e]	Mouse[e]	Systems[f]
Hs.81328	NFkB inhibitor α (IκB-α)	↑	20, 36, 33, 32, 38[g], 39	35, 34[h]	PreB, S49, WEHI, MM, Jurkat, CEM, thymus
Hs.7557	FK506 binding protein 5 (FKBP 51)	↑	20, 36, 37, 33, 39	35	PreB, WEHI, MM, EoL, Jurkat, CEM
Hs.84063	BCL2-like 11 (apoptosis facilitator) – Bim	↑	20, 32, 38[g]	35, 34[h]	PreB, S49, WEHI, CEM, thymus
Hs.420569	GILZ	↑	20, 36, 32	40[i]	PreB, MM, CEM, thymus-2
Hs.111244	HIF-1 responsive RTP801 (dig-2)	↑	38[g]	35, 34[h]	S49, WEHI, thymus, CEM
Hs.146393	Ubiquitin-like domain member 1	↑ and ↓	20	35, 34[h]	PreB, S49, WEHI, thymus
Hs.126608	Glucocorticoid receptor α	↑	32, 38[g], 39	35	PreB, S49, CEM
Hs.362807	Interleukin 7 receptor	↑	33, 32, 38[g]	40[i]	Jurkat, CEM, thymus-2
Hs.50640	Suppressor of cytokine signaling 1 (SOCS1)	↑	37, 32, 38[g], 39		PreB, EoL, CEM
Hs.179526	Thioredoxin interacting protein (TXNIP)	↑	20, 36, 32, 38[g]		PreB, MM, CEM
Hs.422550	Absent in melanoma 1	↑	20, 33, 32, 38[g]		PreB, Jurkat, CEM
Hs.90708	Granzyme A	↑ and ↓	20, 39	35	PreB, S49, WEHI
Hs.13291	Cyclin G2	↑ and ↓	20, 32	35	PreB, S49, CEM
Hs.442669	Glutamine synthase	↑	20, 36	40[i]	PreB, MM, thymus-2
Hs.75231	Solute carrier family 16, member 1 (MCT-1)	↓	20, 33		Pre B, Jurkat, CEM
Hs.6241	PIP-3-kinase, regulatory subunit (p85 α)	↑	37	35	S49, WEHI, EoL
Hs.512712	Tubulin β polypeptide	↓	33	35	WEHI, Jurkat, CEM
M99054	Acid phosphatase type 5	↑		35, 34[h]	S49, WEHI, thymocytes
Hs.131924	G protein-coupled receptor 65	↑		35, 34[h]	S49, WEHI, thymocytes
D50683	TGF-β II receptor α	↑	36, 33		MM.1S, Jurkat, CEM
Hs.315562	Glutamate-cysteine ligase, modifier subunit	↑ and ↓	33, 20		PreB, Jurkat LS7, CEM
Hs.435051	CDK inhibitor 2D (p19, inhibits CDK4)	↑ and ↓	33, 39		Jurkat, CEM, preB
Hs.118183	Hypothetical protein FLJ22833	↓		35, 34[h]	WEHI, S49, thymus
Hs.282326	Down syndrome critical region gene 1	↑	20, 32, 38[g], 39		PreB, CEM
Hs.73958	Recombination activating gene 1 (RAG 1)	↑ and ↓	20, 32, 38[g], 39		PreB, CEM
Hs.202453	c-myc	↓	33, 32, 38[g]		Jurkat, CEM
Hs.443057	CD53 antigen	↑	20, 32, 38[g]		PreB, CEM
Hs.75462	BTG family, member 2	↑	20, 38[g], 39		PreB, CEM
Hs.42322	Paralemmin 2	↑	20, 38[g], 39		PreB, CEM
Hs.528404	Integrin α 4 (antigen CD49D)	↓	20, 32, 38[g]		PreB, CEM
L19314	Human HRY gene, complete cds	↓	33, 32, 38[g]		Jurkat, CEM

[a] Genes are listed according to the number of systems wherein regulation was observed.
[b] Unigene number (starting with Hs.) or GenBank accession number (all others).
[c] Commonly used gene name.
[d] ↑ and ↓ denote two-fold or greater gene induction or repression, respectively.
[e] References to human or mouse work, respectively.
[f] Cellular systems: *Human*: CEM, various subclones of the CCRF-CEM T-ALL cell line as specified in the respective publications; PreB, PreB-697 B-ALL cells; MM; multiple myeloma cell line MM1s; Jurkat; T-ALL cell lines stably transfected with either rat GR^{wt} or rat GR^{LS7}. *Mouse:* WEHI, WEHI7.2 lymphoma cell line; S49, S49.A2 lymphoma cell line; thymus-1, normal C56BL/6 thymocytes; thymus-2, 18-day fetal thymocytes from C57BL/6 wild type mice or GR2KO mice.
[g] Ref. [38] contains only genes regulated in CEM cells sensitive to GC-induced apoptosis but not those regulated both in GC-sensitive and GC-resistant cells. Thus, such genes may be particularly relevant for cell death induction.
[h] Although 59 genes were regulated, only 7 were reported that were found to be regulated in S49 and WEHI as well [34].
[i] Although many more genes were regulated, only 20 genes were reported in [40], i.e., those most strongly regulated in both mouse strains. Since GR2KO mice are resistant to GC-induced apoptosis, regulation of these genes may not suffice for cell death induction.

the apoptotic machinery by regulating components of the extrinsic or, more likely, intrinsic pathways. The most probable candidate at present is the BH3-only molecule Bim which has been shown to be regulated in almost all systems investigated. Since, in addition to Bim, induction of other pro-apoptotic [35,42] and repression of anti-apoptotic [36,43] Bcl-2 family proteins has also been observed, transcriptional deregulation of the "Bcl-2 rheostat" may play a critical role in GC-induced apoptosis. Alternatively or additionally, GC may induce cell death by interfering with critical survival pathways. The most attractive example of this possibility is the induction of IkB, an inhibitor of the survival transcription factor NFkB.

This situation may be complicated, however, by the presence of over-expressed anti-apoptotic Bcl-2 family members or other pro-survival genes as is frequently seen in leukemic cells. In such instances, GC might still be able to induce cell death by gene (de)regulations that induce cellular distress ("component 2" in Fig. 1). Such regulations, like catabolic effects on metabolism or repression of transcription and translation, may be tolerated as long as they are transient (indicated by a small red arrow in Fig. 1). However, they may compromise cell survival when maintained for a sufficient time as a consequence of GR auto-induction (indicated by a large red arrow in Fig. 1). This category might include the regulation of genes affecting metabolic pathways [32,41],

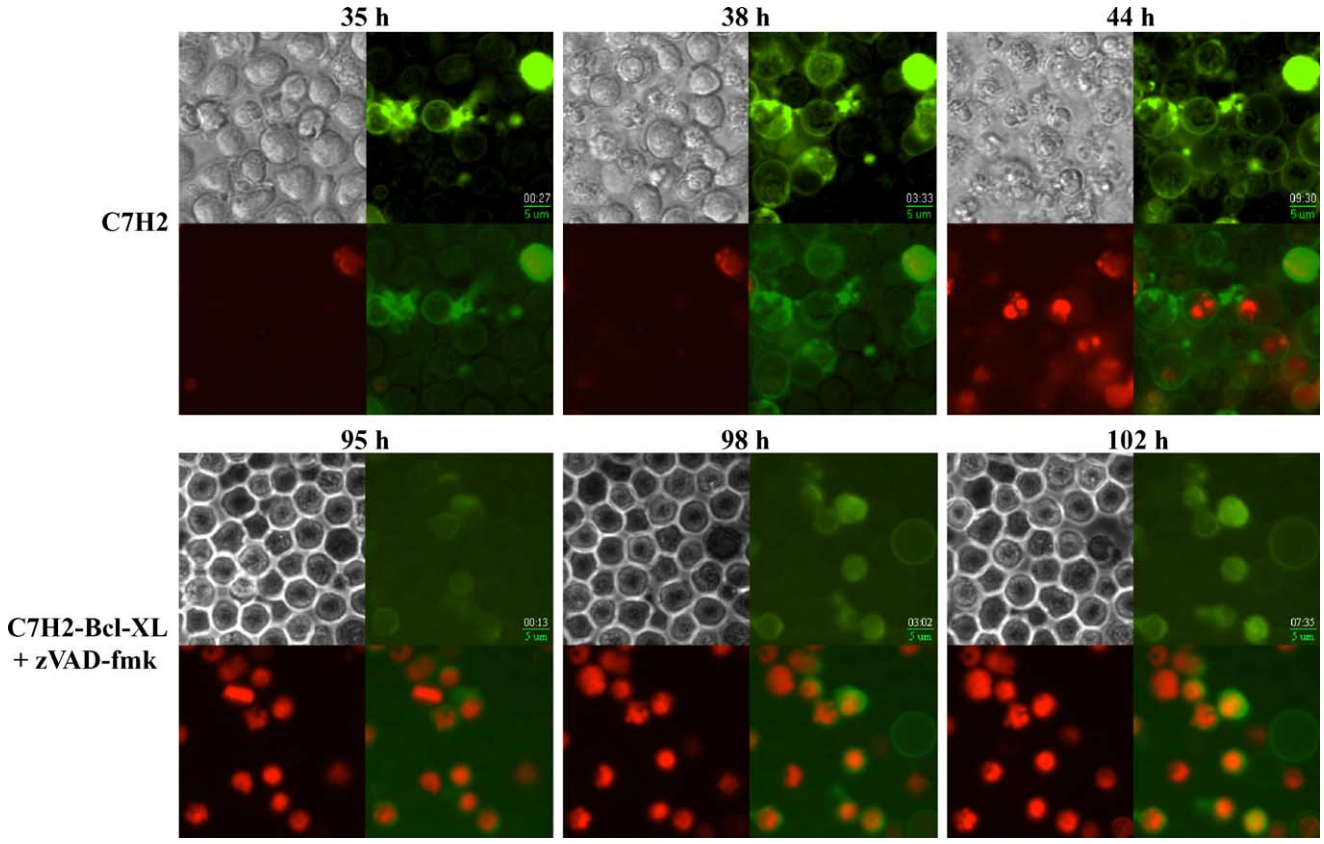

Fig. 2. Different morphology of GC-induced cell death in cells with compromised apoptotic machinery. Untransfected CCRF-CEM-C7H2 were treated with 10^{-7} M dexamethasone (top panels) and compared with CEM-C7H2 cells stably transfected with Bcl-XL and similarly treated with 10^{-7} M dexamethasone but in the presence of 50 μM of the pan-caspase inhibitor zVAD (bottom panels). After 35 h (top row) and 95 h (bottom row), the cells were resuspended in 100 ng/ml of the vital dye propidium iodide (red) and 4 μg/ml Alexa 488-labeled Annexin V (green) and subjected to time-laps video microscopy. Each panel depicts the same cells in phase contrast microscopy (top left), green fluorescence (top right), red fluorescence (bottom left) and an overlay (bottom right). Shown are pictures taken at the beginning (left column), after 3 h (middle column), and at the end (right column) of the time-lapse video microscopy experiment.

general transcription and/or translation [33], production of, or response to, oxygen radicals [44,45], Ca^{2+} fluxes [44,46], or intracellular pH [47] and volume control [48]. Examples from Table 1 that support this concept include the induction of the thioredoxin inhibitor, TXNIP, or repression of the lactate transporter, MCT-1. The former might contribute to increased oxidative stress, the latter to metabolic alterations, pH changes and/or disturbed volume control. This cell death form, which occurs in the presence of a compromised apoptotic machinery, may invoke a more necrotic morphology. In support, high levels of transgenic anti-apoptotic Bcl-2 proteins, even in combination with saturating amounts of the pan-caspase inhibitor z-VAD, failed to restore viability in GC-treated CCRF-CEM T-ALL cells in the continuous presence of the drug (C. Ploner et al., in preparation). The resulting cell death was, however, markedly delayed and showed altered morphology, including reduction of DNA fragmentation, membrane blebbing and formation of apoptotic bodies, but increased occurrence of cells permeable for vital dyes, as shown by time-lapse video microscopy (Fig. 2). Thus, there may be a continuous transition between two cell death forms, one rapid and showing typical apoptotic features in cells with low levels of Bcl-2 (and/or other anti-apoptotic molecules) and another retarded form with more necrotic characteristics in cells with high levels of such proteins. The latter may be critically dependent upon GR auto-induction.

3. Mechanisms of resistance to GC-induced apoptosis

A large number of possible molecular mechanisms for GC resistance can be envisaged along the signal transduction pathways triggered by GC [5,12,49–51]. Conceptually, they may be grouped into “upstream” and “downstream” mechanisms (Fig. 3). The former encompass the GR, its ligand and GR-associated proteins that control its function, and can potentially affect most, if not all, GC effects. The latter interfere with only individual GC responses and include cross-talks with interfering pathways, mutations in, or lack of responsiveness of, downstream targets of the GR, etc.

To experimentally address possible resistance mechanisms in ALL cells, we generated an in vitro resistance model consisting of panels of GC-resistant and -sensitive subclones of the CCRF-CEM-C7H2 lymphoblastic leukemia

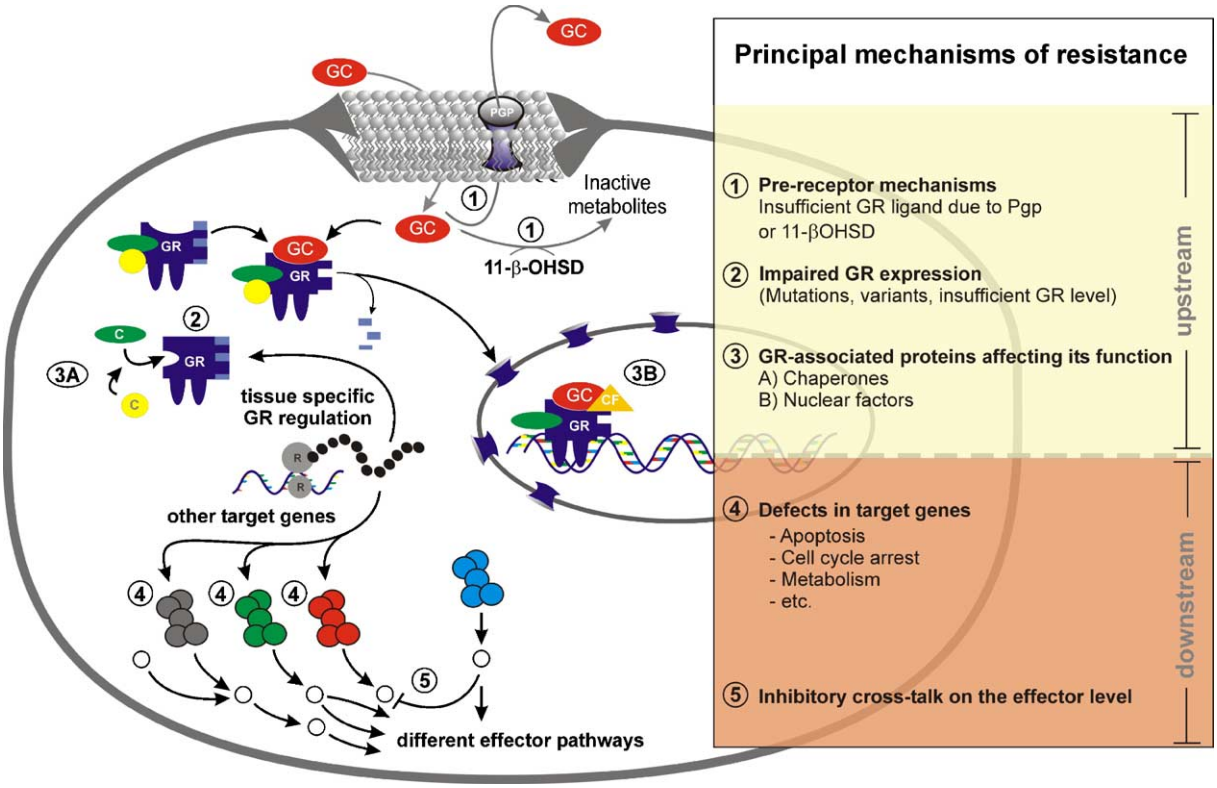

Fig. 3. Principal mechanisms of GC resistance. Possible resistance mechanisms were organized along the GC signaling pathway: "upstream" mechanisms include (1) "prereceptor defects, (2) structural and regulatory abnormalities in GR expression, and (3) deficiencies in GR-associated proteins, and may affect most, if not all, GC responses. "Downstream" mechanisms encompass (4) defects in components of the specific response pathway or (5) cross-talk from other signaling pathways that interfere with and antagonize a given GC response. Abbreviations: 11β-OHSD, 11β-hydroxysteroid dehydrogenase; C, chaperones; GC, glucocorticoid; GR, glucocorticoid receptor; Pgp, *p*-glycoprotein; R, ribosome. Taken from Ref. [49] with permission.

cell line obtained by limiting dilution cloning in the presence or absence of 10^{-7} M dexamethasone. These cell lines are currently used as "discriminatory pairs" in comparative expression profiling. To address a possible role of GR autoinduction (and lack thereof), we determined the expression of GR mRNA in these cell lines prior to, and 6–8 and 24 h after exposure to 10^{-7} M dexamethasone using quantitative "real-time" RT-PCR. As a representative downstream target of the GR, we further quantified expression levels of GILZ, a GC-induced leuzine zipper protein implicated in the antagonistic effect of GC on T-cell receptor-induced apoptosis in thymocytes [52,53]. As depicted in Fig. 4, all GC-sensitive lines, like parental C7H2 cells, markedly induced both GR and GILZ mRNAs after 6–8 and 24 h. In sharp contrast, the resistant lines failed to regulate GR and GILZ to the same extent. In a collaborative effort (J. Irving and A. Hall, Northern Cancer Research Institute, Newcastle upon Tyne; W. Parson, R. Mühlbacher, Institute of Forensic Medicine, Innsbruck; M. Erdel, C. Vaut, Institute of Biology and Human Genetics, Innsbruck), we are currently applying denaturing HPLC, sequencing, DNA fingerprinting and chromosomal analyses to delineate the molecular mechanisms underlying the above phenomenon. Preliminary experiments suggest that both GR mutations/deletion and regulatory mechanisms account for resistance development. Thus, at least in this model system, GC resistance is strongly associated with a failure to autoinduce GR expression.

To investigate whether this form of GC resistance is also observed in other ALL systems, we further delineated the molecular mechanism for GC resistance in the widely used Jurkat T-ALL cell line [54]. GC-resistant Jurkat cells, like their GC-sensitive counterpart CCRF-CEM [55], are heterozygotes at the GR locus ($GR^{R477H/wt}$). The mutation, although proficient in ligand binding and nuclear translocation, confers inability to transactivate and transrepress gene expression from corresponding reporter constructs. However, like the loss-of-function mutation in CCRF-CEMs (L753F), it is not dominant negative and hence does not fully explain GC resistance. But unlike GC-sensitive CCRF-CEM cells, GC-resistant Jurkats fail to auto-induce their GR. Thus, it appears that "upstream mechanisms", in particular structural and/or regulatory defects in GR expression, might be a frequent cause of GC resistance in human leukemia cell lines. GC resistance in leukemia patients requires both inhibition of GC-induced cell cycle arrest and apoptosis. Since these two processes, at least in ALL cell lines, follow distinct pathways [56], interfering with GR expression might be the most efficient means to escape the antileukemic effects of GC.

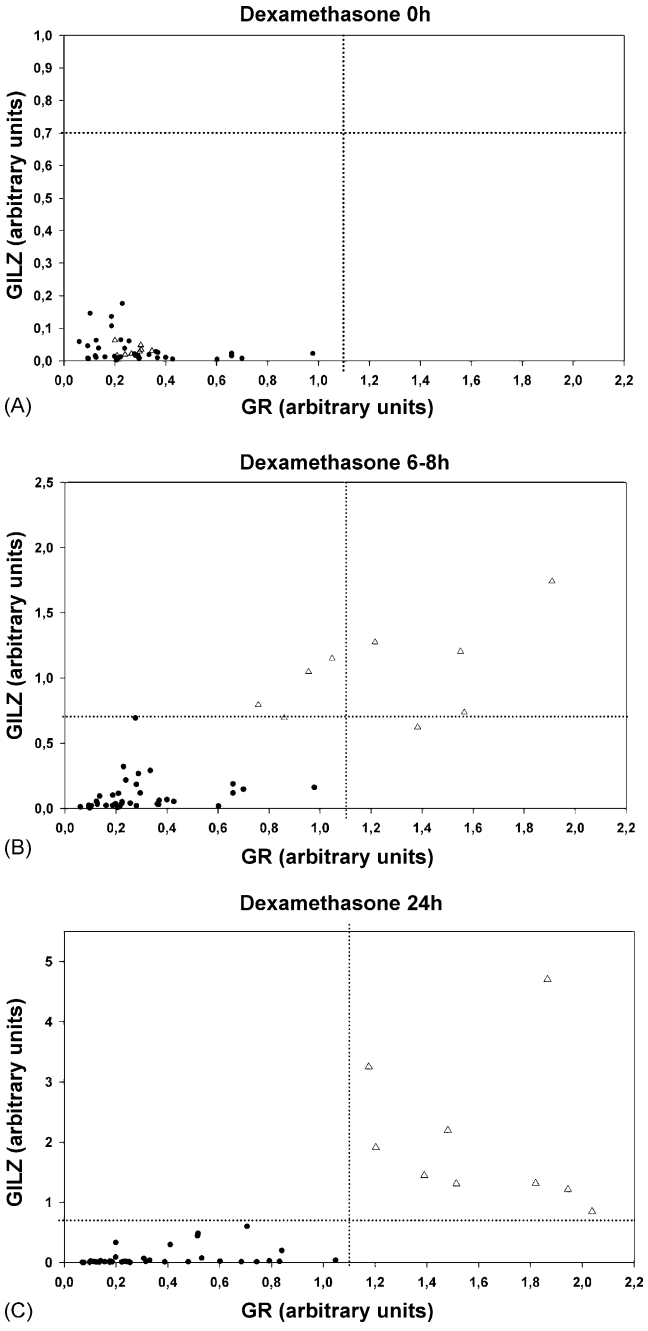

Fig. 4. GC resistance is associated with impaired GR and/or GILZ induction. Nine GC-sensitive (open triangles) and 39 GC-resistant (black circles) subclones of the GC-sensitive CCRF-CEM-C7H2 human T-ALL cell line were treated with 10^{-7} M dexamethasone for the indicated times and their RNA subjected to quantitative "real-time" PCR to assess the levels of GR and GILZ in relation to TATA-box-binding protein used as a control. Note that the scale of the *Y*-axis is different in the three plots for better data visualization. After 24-h dexamethasone exposure all GC-sensitive, but none of the GC-resistant, cells shift to the top right quadrant. All cells (including the GC-sensitive ones) are alive at 24 h, apoptosis starts around 36 h in GC-sensitive cells.

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References

[1] J.D. Ashwell, F.W. Lu, M.S. Vacchio, Glucocorticoids in T cell development and function, Annu. Rev. Immunol. 18 (2000) 309–345.
[2] J.A. Cidlowski, K.L. King, R.B. Evans-Storms, J.W. Montague, C.D. Bortner, F.M. Hughes Jr., The biochemistry and molecular biology of glucocorticoid-induced apoptosis in the immune system, Recent Prog. Horm. Res. 51 (1996) 457–490.
[3] M. Jondal, A. Pazirandeh, S. Okret, A role for glucocorticoids in the thymus? Trends Immunol. 22 (2001) 185–186.
[4] P.S. Gaynon, A.L. Carrel, Glucocorticosteroid therapy in childhood acute lymphoblastic leukemia, Adv. Exp. Med. Biol. 457 (1999) 593–605.
[5] W.J. Tissing, J.P. Meijerink, M.L. den Boer, R. Pieters, Molecular determinants of glucocorticoid sensitivity and resistance in acute lymphoblastic leukemia, Leukemia 17 (2003) 17–25.
[6] C.W. Distelhorst, Recent insights into the mechanism of glucocorticosteroid-induced apoptosis, Cell Death Differ. 9 (2002) 6–19.
[7] S. Greenstein, K. Ghias, N.L. Krett, S.T. Rosen, Mechanisms of glucocorticoid-mediated apoptosis in hematological malignancies, Clin. Cancer Res. 8 (2002) 1681–1694.
[8] S.L. Planey, G. Litwack, Glucocorticoid-induced apoptosis in lymphocytes, Biochem. Biophys. Res. Commun. 279 (2000) 307–312.
[9] C. Riccardi, O. Zollo, G. Nocentini, S. Bruscoli, A. Bartoli, F. D'Adamio, L. Cannarile, D. Delfino, E. Ayroldi, G. Migliorati, Glucocorticoid hormones in the regulation of cell death, Therapie 55 (2000) 165–169.
[10] E.B. Thompson, Special topic: apoptosis, Annu. Rev. Physiol. 60 (1998) 525–532.
[11] S. Schmidt, J. Rainer, C. Ploner, E. Presul, S. Riml, R. Kofler, Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance, Cell Death Differ. 11 (2004) S45–55.
[12] K. Renner, M.J. Ausserlechner, R. Kofler, A conceptual view on glucocorticoid-induced apoptosis, cell cycle arrest and glucocorticoid resistance in lymphoblastic leukemia, Curr. Mol. Med. 3 (2003) 707–717.
[13] M.O. Hengartner, The biochemistry of apoptosis, Nature 407 (2000) 770–776.
[14] X. Wang, The expanding role of mitochondria in apoptosis, Genes Dev. 15 (2001) 2922–2933.
[15] D.W. Nicholson, N.A. Thornberry, Apoptosis: life and death decisions, Science 299 (2003) 214–215.

[16] P.H. Krammer, CD95's deadly mission in the immune system, Nature 407 (2000) 789–795.
[17] J. Yuan, B.A. Yankner, Apoptosis in the nervous system, Nature 407 (2000) 802–809.
[18] S. Geley, B.L. Hartmann, K. Kapelari, A. Egle, A. Villunger, D. Heidacher, R. Greil, B. Auer, R. Kofler, The interleukin 1b-converting enzyme inhibitor crmA prevents Apo1/fas- but not glucocorticoid-induced poly(ADP-ribose) polymerase cleavage and apoptosis in lymphoblastic leukemia cells, FEBS Lett. 402 (1997) 36–40.
[19] K.G.C. Smith, A. Strasser, D.L. Vaux, CrmA expression in T lymphocytes of transgenic mice inhibits CD95 (Fas/APO-1)-transduced apoptosis, but does not cause lymphadenopathy or autoimmune disease, EMBO J. 15 (1996) 5167–5176.
[20] S.L. Planey, M.T. Abrams, N.M. Robertson, G. Litwack, Role of apical caspases and glucocorticoid-regulated genes in glucocorticoid-induced apoptosis of pre-B leukemic cells, Cancer Res. 63 (2003) 172–178.
[21] B.L. Hartmann, S. Geley, M. Löffler, R. Hattmannstorfer, E.M.C. Strasser-Wozak, B. Auer, R. Kofler, Bcl-2 interferes with the execution phase, but not upstream events, in glucocorticoid-induced leukemia apoptosis, Oncogene 18 (1999) 713–719.
[22] F. Cecconi, G. Alvarez-Bolado, B.I. Meyer, K.A. Roth, P. Gruss, Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development, Cell 94 (1998) 727–737.
[23] H. Yoshida, Y.Y. Kong, R. Yoshida, A.J. Elia, A. Hakem, R. Hakem, J.M. Penninger, T.W. Mak, Apaf1 is required for mitochondrial pathways of apoptosis and brain development, Cell 94 (1998) 739–750.
[24] R. Hakem, A. Hakem, G.S. Duncan, J.T. Henderson, M. Woo, M.S. Soengas, A. Elia, J.L. De la Pompa, D. Kagi, W. Khoo, J. Potter, R. Yoshida, S.A. Kaufman, S.W. Lowe, J.M. Penninger, T.W. Mak, Differential requirement for caspase 9 in apoptotic pathways in vivo, Cell 94 (1998) 339–352.
[25] K. Kuida, T.F. Haydar, C.Y. Kuan, Y. Gu, C. Taya, H. Karasuyama, M.S.S. Su, P. Rakic, R.A. Flavell, Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9, Cell 94 (1998) 325–337.
[26] J.C. Rathmell, T. Lindsten, W.X. Zong, R.M. Cinalli, C.B. Thompson, Deficiency in Bak and Bax perturbs thymic selection and lymphoid homeostasis, Nat. Immunol. 3 (2002) 932–939.
[27] P. Bouillet, D. Metcalf, D.C. Huang, D.M. Tarlinton, T.W. Kay, F. Kontgen, J.M. Adams, A. Strasser, Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity, Science 286 (1999) 1735–1738.
[28] A. Villunger, E.M. Michalak, L. Coultas, F. Mullauer, G. Bock, M.J. Ausserlechner, J.M. Adams, A. Strasser, p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa, Science 302 (2003) 1036–1038.
[29] C.M. Knudson, K.S.K. Tung, W.G. Tourtellotte, G.A.J. Brown, S.J. Korsmeyer, Bax-deficient mice with lymphoid hyperplasia and male germ cell death, Science 270 (1995) 96–99.
[30] X.M. Yin, K. Wang, A. Gross, Y. Zhao, S. Zinkel, B. Klocke, K.A. Roth, S.J. Korsmeyer, Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis, Nature 400 (1999) 886–891.
[31] S. Geley, M. Fiegl, B.L. Hartmann, R. Kofler, Genes mediating glucocorticoid effects and mechanisms of their regulation, Rev. Physiol. Biochem. Pharmacol. 128 (1996) 1–97.
[32] M. Tonko, M.J. Ausserlechner, D. Bernhard, A. Helmberg, R. Kofler, Gene expression profiles of proliferating versus G1/G0 arrested human leukemia cells suggest a mechanism for glucocorticoid-induced apoptosis, FASEB J. 15 (2001) 693–699.
[33] P. Obexer, U. Certa, R. Kofler, A. Helmberg, Expression profiling of glucocorticoid-treated T-ALL cell lines: rapid repression of multiple genes involved in RNA, protein and nucleotide synthesis, Oncogene 20 (2001) 4324–4336.
[34] Z. Wang, M.H. Malone, M.J. Thomenius, F. Zhong, F. Xu, C.W. Distelhorst, Dexamethasone-induced gene 2 (dig2) is a novel pro-survival stress gene induced rapidly by diverse apoptotic signals, J. Biol. Chem. 278 (2003) 27053–27058.
[35] Z. Wang, M.H. Malone, H. He, K.S. McColl, C.W. Distelhorst, Microarray analysis uncovers the induction of the pro-apoptotic BH3-only protein Bim in multiple models of glucocorticoid induced apoptosis, J. Biol. Chem. 278 (2003) 23861–23867.
[36] D. Chauhan, D. Auclair, E.K. Robinson, T. Hideshima, G. Li, K. Podar, D. Gupta, P. Richardson, R.L. Schlossman, N. Krett, L.B. Chen, N.C. Munshi, K.C. Anderson, Identification of genes regulated by dexamethasone in multiple myeloma cells using oligonucleotide arrays, Oncogene 21 (2002) 1346–1358.
[37] S. Chauhan, C.H. Leach, S. Kunz, J.W. Bloom, R.L. Miesfeld, Glucocorticoid regulation of human eosinophil gene expression, J. Steroid Biochem. Mol. Biol. 84 (2003) 441–452.
[38] M.S. Webb, A.L. Miller, B.H. Johnson, Y. Fofanov, T. Li, T.G. Wood, E.B. Thompson, Gene networks in glucocorticoid-evoked apoptosis of leukemic cells, J. Steroid Biochem. Mol. Biol. 85 (2003) 183–193.
[39] N.L. Yoshida, T. Miyashita, U.M.M. Yamada, J.C. Reed, Y. Sugita, T. Oshida, Analysis of gene expression patterns during glucocorticoid-induced apoptosis using oligonucleotide arrays, Biochem. Biophys. Res. Commun. 293 (2002) 1254–1261.
[40] P.R. Mittelstadt, J.D. Ashwell, Disruption of glucocorticoid receptor exon 2 yields a ligand-responsive C-terminal fragment that regulates gene expression, Mol. Endocrinol. 17 (2003) 1534–1542.
[41] R. Kofler, The molecular basis of glucocorticoid-induced apoptosis of lymphoblastic leukemia cells, Histochem. Cell Biol. 114 (2000) 1–7.
[42] J. Han, C. Flemington, A.B. Houghton, Z. Gu, G.P. Zambetti, R.J. Lutz, L. Zhu, T. Chittenden, Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 11318–11323.
[43] F. Casale, R. Addeo, V. D'Angelo, P. Indolfi, V. Poggi, C. Morgera, S. Crisci, M.T. Di Tullio, Determination of the in vivo effects of prednisone on Bcl-2 family protein expression in childhood acute lymphoblastic leukemia, Int. J. Oncol. 22 (2003) 123–128.
[44] A. Fernandez, J. Kiefer, L. Fosdick, D.J. McConkey, Oxygen radical production and thiol depletion are required for Ca^{2+}-mediated endogenous endonuclease activation in apoptotic thymocytes, J. Immunol. 155 (1995) 5133–5139.
[45] M.E. Tome, M.M. Briehl, Thymocytes selected for resistance to hydrogen peroxide show altered antioxidant enzyme profiles and resistance to dexamethasone-induced apoptosis, Cell Death Differ. 8 (2001) 953–961.
[46] C.W. Distelhorst, G. Dubyak, Role of calcium in glucocorticosteroid-induced apoptosis of thymocytes and lymphoma cells: resurrection of old theories by new findings, Blood 91 (1998) 731–734.
[47] S. Matsuyama, J.C. Reed, Mitochondria-dependent apoptosis and cellular pH regulation, Cell Death Differ. 7 (2000) 1155–1165.
[48] M. Gomez-Angelats, C.D. Bortner, J.A. Cidlowski, Cell volume regulation in immune cell apoptosis, Cell Tissue Res. 301 (2000) 33–42.
[49] R. Kofler, S. Schmidt, A. Kofler, M.J. Ausserlechner, Resistance to glucocorticoid-induced apoptosis in lymphoblastic leukemia, J. Endocrinol. 178 (2003) 19–27.
[50] E.G. Haarman, G.J. Kaspers, A.J. Veerman, Glucocorticoid resistance in childhood leukaemia: mechanisms and modulation, Br. J. Haematol. 120 (2003) 919–929.
[51] M.J. Schaaf, J.A. Cidlowski, Molecular mechanisms of glucocorticoid action and resistance, J. Steroid Biochem. Mol. Biol. 83 (2002) 37–48.
[52] F. D'Adamio, O. Zollo, R. Moraca, E. Ayroldi, S. Bruscoli, A. Bartoli, L. Cannarile, G. Migliorati, C. Riccardi, A new dexamethasone-induced gene of the leucine zipper family protects T lymphocytes from TCR/CD3-activated cell death, Immunity 7 (1997) 803–812.
[53] C. Riccardi, M.G. Cifone, G. Migliorati, Glucocorticoid hormone-induced modulation of gene expression and regulation of T-cell death: role of GITR and GILZ, two dexamethasone-induced genes, Cell Death Differ. 6 (1999) 1182–1189.

[54] S. Riml, S. Schmidt, M.J. Ausserlechner, S. Geley, R. Kofler, Glucocorticoid receptor heterozygosity combined with lack of receptor auto-induction causes glucocorticoid resistance in Jurkat acute lymphoblastic leukemia cells, Cell Death Differ. 11 (2004) S65–72.
[55] J. Ashraf, E.B. Thompson, Identification of the activation-labile gene: a single point mutation in the human glucocorticoid receptor presents as two distinct receptor phenotypes, Mol. Endocrinol. 7 (1993) 631–642.
[56] M.J. Ausserlechner, P. Obexer, G. Böck, S. Geley, R. Kofler, Cyclin D3 and c-myc control glucocorticoid-induced cell cycle arrest but not apoptosis in lymphoblastic leukemia cells, Cell Death Differ. 11 (2004) 165–174.

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Review

Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance

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Abstract

The ability of glucocorticoids (GC) to efficiently kill lymphoid cells has led to their inclusion in essentially all chemotherapy protocols for lymphoid malignancies. This review summarizes recent findings related to the molecular basis of GC-induced apoptosis and GC resistance, and discusses their potential clinical implications. Accumulating evidence suggests that GC may induce cell death via different pathways resulting in apoptotic or necrotic morphologies, depending on the availability/responsiveness of the apoptotic machinery. The former might result from regulation of typical apoptosis genes such as members of the Bcl-2 family, the latter from detrimental GC effects on essential cellular functions possibly perpetuated by GC receptor (GR) autoinduction. Although other possibilities exist, GC resistance might frequently result from defective GR expression, perhaps the most efficient means to target multiple antileukemic GC effects. Numerous novel drug combinations are currently being tested to prevent resistance and improve GC efficacy in the therapy of lymphoid malignancies.
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Abbreviations: ALL, acute lymphoblastic leukemia; GC, glucocorticoid(s); GR, glucocorticoid receptor

Introduction

Glucocorticoid (GC)-induced apoptosis is a phenomenon of considerable biological and clinical significance. Biologically, it has been implicated in the generation of the immune repertoire and the regulation of immune responses,[1–3] and clinically it has been exploited in the therapy of lymphoid malignancies.[4] In this review, we summarize current concepts regarding the molecular mechanism of this GC response and resistance against it, and discuss the potential clinical impact of emerging knowledge in this field. Space limitations precluded a complete reference to the large body of literature and we apologize to our colleagues for often citing reviews and exemplary work rather than all relevant original publications. To put our topic into perspective, we first provide a short introduction to the multitude of GC effects and their basic mechanism of action, and briefly discuss distinct forms of cell death as they relate to the topic of this review. The subsequent section summarizes the molecular components of GC-triggered death pathway, beginning with the role of glucocorticoid receptor (GR) expression, and subsequently addressing the controversial question of whether transactivation or transrepression is required, outlining GC-regulated genes as revealed by gene expression profiling studies, and finally providing a tentative model for this death response. The next section on Mechanisms of resistance to GC-induced apoptosis deals with GC resistance with particular emphasis on mechanisms acting at the level of the GR. The clinical significance of these phenomena and issues related to exploiting the true therapeutic potential of GC in novel combination protocols are topics of the last section.

Pleiomorphic effects of GC

Depending on a number of modulating factors, such as GC type and concentration, extracellular milieu, intracellular context, etc, GC and their analogues mediate a variety of effects on mammalian cells and entire organisms. These include pronounced effects on metabolism that primarily lead to catabolism of proteins, lipids and carbohydrates. GC increase blood sugar levels, cause osteoporosis, and play an important role in the stress response. They further repress cell cycle progression in a number of systems including acute lymphoblastic leukemia (ALL).[5,6] At least in therapeutic concentrations, GC are strongly immunosuppressive and anti-inflammatory,[7] which has made them one of the most frequently prescribed drugs worldwide.

Pertinent to this review, GC influence survival in many tissues in a cell-type-specific manner. As documented by over 2200 publications in the PubMed database and summarized in numerous recent reviews,[5,8–13] GC induce massive apoptosis in certain cells of the lymphoid lineage, particularly immature thymocytes and ALL cells, and the latter has been exploited in the therapy of lymphoid malignancies.[4] GC have further been reported to induce cell death (alone or in

combination with other death inducers) in some nonlymphoid tissues and cells such as bone,[14] hippocampus,[15] eosinophils,[16] fibroblasts[17] and certain cancer cells.[18] Interestingly, GC support survival in erythroblasts,[19] neutrophils[20] and several nonhematologic tissues such as mammary gland, ovary, liver and fibroblasts (reviewed in Amsterdam and Sasson[21]). Such prosurvival effects may become clinically relevant when they interfere with the effect of chemotherapeutics.[22] Depending upon the circumstances, GC both triggered cell death and supported survival in some cells,[23] further documenting the pro- and antiapoptotic potential of this hormone.

General mode of action of GC

Although receptor-independent effects may occur at very high concentrations (presumably through membrane perturbation[24]), most, if not all, effects of GC at physiologic or therapeutic levels are mediated by the GC receptor (GR, Figure 1). The GR is a ligand-activated transcription factor of the nuclear receptor family (steroid receptor subfamily, comprised of seven members: estrogen receptor α and β, estrogen-related receptors 1 and 2, and the receptors for mineralcorticoids, androgens and progesterone).[25] It resides in the cytoplasm in a multiprotein complex.[26] Upon ligand binding, the GR dissociates from at least some of its binding proteins and translocates into the nucleus to induce or repress the expression of a plethora of genes identified by conventional gene searches (reviewed in Geley *et al.*)[27] or microarray analyses.[28–38] Gene induction is mediated via GR interaction with conserved response DNA elements (GC responsive elements, GREs: GGTACANNNTGTTCT[25]), whereas gene repression occurs through negative GREs, protein–protein interaction with other sequence-specific transcription factors, competition for coactivators and other mechanisms (reviewed in Laudet and Gronemeyer[25] and Geley *et al.*[27]). While this is probably the mechanism underlying most GC effects, these hormones can exert more immediate (20–30 min), presumably nongenomic but still GR-dependent, effects, the mechanism of which is less well understood.[24,39] In addition to the well-characterized cytoplasmic/nuclear GR, a membrane-associated species was reported,[40] but its existence and possible significance remained controversial.

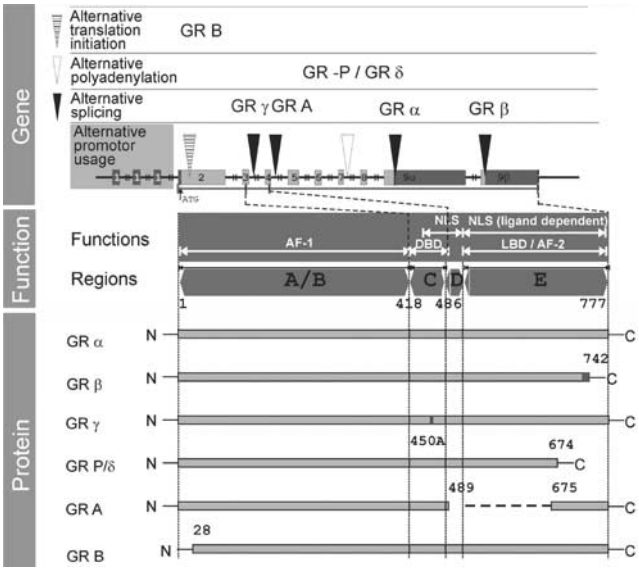

Figure 1 The human GR gene and the known GR variants. The top panel summarizes the genomic organization of the GR gene (NR3C1) on chromosome 5q31/32 and depicts various molecular mechanisms leading to six variant GR transcripts. Their schematic protein structure is given in the bottom panel. The middle panel relates the intron/exon structure and protein regions to their presumed function. Note that the GR gene has five published[125] and at least four additional untranslated exons 1 (Presul *et al.*, in preparation) of unknown significance. a/b, c, d and e refer to protein regions of nuclear receptors, the numbers denote amino-acid positions.[25] AF, sequences implied in transactivation; DBD, DNA-binding doman; LBD, ligand-binding domain; NLS, nuclear localization sequence

Cell death forms: apoptosis, necrosis and the 'in betweens'

Classically, two major cell death forms have been distinguished: (1) apoptosis, an active and ordered form of cellular suicide characterized by a number of morphologic criteria such as cell shrinkage, membrane blebbing, formation of apoptotic bodies, DNA cleavage and condensation, caspase activation, phosphatidylserine expression on the outer cell membrane, etc., and (2) necrosis or accidental cell death with membrane rupture and subsequent release of potentially inflammatory cell constituents into the surrounding tissue. Regarding apoptosis, two major signaling pathways have been described: the 'extrinsic' pathway that is initiated by ligand-mediated activation of membrane death receptors, and the 'intrinsic' pathway that is controlled by members of the Bcl-2 family and mitochondria-derived proteins. In the context of this review, we suggest to further differentiate between two conceptually distinct types of apoptotic cell death (Figure 2). In the first, apoptosis occurs in an entirely healthy cell because the apoptosis machinery has been activated (e.g., by specific

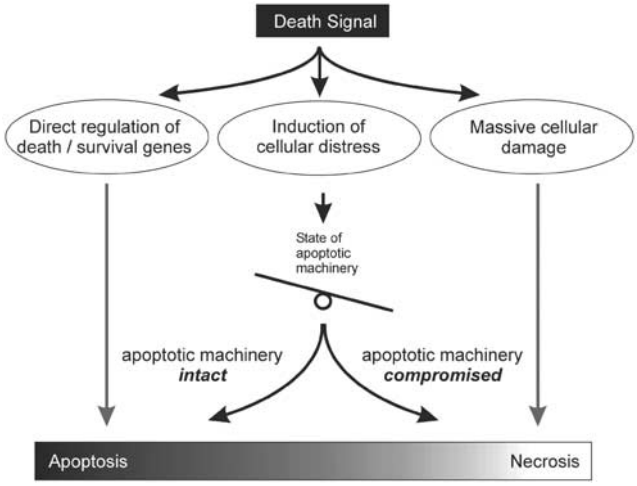

Figure 2 Hypothetical classification of cell death forms. In this model, death signals induce cellular demise in three different ways: first, by directly regulating crucial death or survival genes leading to apoptotic cell death; second, through cellular distress that might lead to either apoptosis or necrosis depending upon the availability of the apoptotic machinery; third, via massive cellular damage leading to necrotic cell death (for other details see text)

regulation of its key components). In the second, the apoptosis machinery is activated because the cell recognizes, and responds to, a harmful and potentially deadly insult. In the first, viability can be restored by interference with apoptosis effectors, which is not the case in the second, because blocking apoptosis does not affect the primary, and ultimately deadly, insult. In such instances, that is, when the cell is damaged but the apoptotic machinery is completely or partially compromised, the resulting cell death may adopt a more or less necrotic morphology. As discussed below in more detail, GC may induce cell death via several of these mechanisms.

Molecular mechanisms of GC-induced apoptosis

Initiation of the apoptotic response: role of the GR and its regulation

GC-induced apoptosis is initiated by, and strictly dependent upon, the interaction of GC with its receptor, the GR. The requirement for the receptor has been shown in thymocytes from genetically modified mice[41] and human ALL cell lines[42] with mutated GR, and by conferring GC sensitivity to GC-resistant ALL cell lines by GR transgenesis.[43,44] Moreover, the level of GR expression is a critical determinant for GC sensitivity, as suggested by studies in transgenic mice with increased[45] or decreased[46] GR expression, human T-ALL cell lines with different GR levels[43] and GC-sensitive and -resistant multiple myeloma lines.[34] However, GR expression at the onset of the response may represent only part of this mechanism: Removal of GC within the first 24 h prevents cell death in ALL cells,[47] suggesting that sufficient GR levels need to be maintained for a considerable time. GR expression, however, is subject to negative feedback regulation, at least in cells not undergoing GC-induced apoptosis.[48] In contrast, in cells sensitive to the cytolytic effect of GC, evidence for GR autoinduction has been provided: In multiple myeloma lines, GC sensitivity and resistance correlated with induction[49] and repression[50] of GR mRNA, respectively. Elegant experiments exploiting tetracycline-regulated GR expression showed requirement of GR autoinduction for GC-induced apoptosis in CCRF-CEM derivatives,[51] and impaired GR autoinduction was observed in GC-resistant, but not in GC-sensitive, subclones of the same ALL model.[52] Moreover, Jurkat T-ALL cells, which, like GC-sensitive CCRF-CEM T-ALL cells, carry one wild-type and one mutated GR allele, are GC resistant and fail to autoinduce their GR.[53] Maintaining high GR levels through constitutive expression of transgenic GR leads to GC sensitivity in these cells.[44] Thus, at least in leukemia cell lines, maintenance of sufficient GR levels throughout a critical phase of the response appears mandatory (although not necessarily sufficient) for cell death induction, and this might be accomplished by GR autoinduction.

Gene transactivation, transrepression or both?

Although it is widely accepted that cell death induction by GC results from alterations in gene expression, it is still controversial whether it requires gene transactivation, transrepression or both. Mice carrying a dimerization deficient GR (GR^{dim} 'knockin' mice) are deficient in GC-induced thymocyte apoptosis,[41] suggesting that transactivation is required (although not necessarily sufficient in itself). A similar conclusion was derived from studies in GR-deficient S49 mouse thymoma cells transfected with N-terminal-deleted GR constructs.[54] In contrast, GC sensitivity could be restored in GC-resistant Jurkat[44] and CEM[55] human T-ALL cells by constitutive expression of transactivation-deficient GR mutants, suggesting that transrepression alone was sufficient for cell death induction (although a potential requirement for transactivation-dependent GR autoinduction would not have been detected in these studies because the GR was expressed from a strong constitutive promoter). More recently, we generated several transgenic subclones of the GR-deficient CEM-C7R1 cell line[42] with different expression levels of either GR^{wt} or GR^{dim} and found that the mutant conferred GC sensitivity only if expressed at considerably higher levels than the wild type (S Riml, in preparation). At these levels, the remaining transactivation potential of the mutant GR might suffice to induce critical target genes, a hypothesis currently being tested by comparative expression profiling. Thus, neither transactivation nor transrepression has been conclusively ruled out by the above studies, and it is possible that both mechanisms contribute to GC-induced cell death.

GC-regulated genes responsible for cell death induction

The key question of which GC-regulated genes are responsible for triggering cell death has been addressed by a number of classical gene search approaches (reviewed in Geley *et al*.[27]) without providing a generally accepted answer. Recent microarray-based expression profiling of cells undergoing GC-induced cell death has considerably increased the number of potential candidate genes.[29–38] An ongoing detailed bioinformatic meta-analysis of these publications (Schmidt *et al*., in preparation) revealed that not a single gene was found to be regulated in all eight investigated biological systems (cutoff: more than two-fold), and only a few appeared in three or more systems and/or publications (Table 1). Altogether, some 900 different genes were reported as GC regulated, but of these only $\sim$70 appeared in more than one publication. Although this small number might result from the use of arrays with only partially overlapping gene composition, technical or bioinformatic problems, and the way regulated genes were reported (in some papers only a selection of all regulated genes), it still suggests that a distinct set of genes might be regulated in different cell systems and experimental conditions. This raises the possibility that multiple, cell-context-dependent mechanisms rather than a conserved canonical pathway may lead to GC-induced cell death. However, since only about one-third of the human genome has been studied thus far, important genes may have been missed and a shared pathway may eventually be revealed.

Given the limitations noted above, the gene list in Table 1 is far from complete and thus cannot exclude additional

Table 1 Genes regulated by GC in cells prone to GC-induced apoptosis[a]

Identifier[b]	Description[c]	Reg[d]	Human[e]	Mouse[e]	Systems[f]
Hs.81328	NFkB inhibitor α (IκB-α)	↑	31,34,30,29,36[g],37	33, 32[h]	PreB, S49, WEHI, MM, Jurkat, CEM, thymus
Hs.7557	FK506-binding protein 5 (FKBP 51)	↑	31,34,35,30,37	33	PreB, WEHI, MM, EoL, Jurkat, CEM
Hs.84063	BCL2-like 11 (apoptosis facilitator) – Bim	↑	31,29,36[g]	33, 32[h]	PreB, S49, WEHI, CEM, thymus
Hs.420569	GILZ	↑	31,34,29	38[i]	PreB, MM, CEM, thymus-2
Hs.111244	HIF-1 responsive RTP801 (dig-2)	↑	36[g]	33, 32[h]	S49, WEHI, thymus, CEM
Hs.146393	Ubiquitin-like domain member 1	↑&↓	31	33, 32[h]	PreB, S49, WEHI, thymus
Hs.126608	Glucocorticoid receptor α	↑	29,36[g],37	33	PreB, S49, CEM
Hs.362807	Interleukin 7 receptor	↑	30,29,36[g]	38[i]	Jurkat, CEM, thymus-2
Hs.50640	Suppressor of cytokine signaling 1 (SOCS1)	↑	35,29,36[g],37		PreB, EoL, CEM
Hs.179526	Thioredoxin interacting protein (TXNIP)	↑	31,34,29,36[g]		PreB, MM, CEM
Hs.422550	Absent in melanoma 1	↑	31,30,29,36[g]		PreB, Jurkat, CEM
Hs.90708	Granzyme A	↑&↓	31,37	33	PreB, S49, WEHI
Hs.13291	Cyclin G2	↑&↓	31,29	33	PreB, S49, CEM
Hs.442669	Glutamine synthase	↑	31,34	38[i]	PreB, MM, thymus-2
Hs.75231	Solute carrier family 16, member 1 (MCT-1)	↓	31,30		Pre B, Jurkat, CEM
Hs.6241	PIP-3-kinase, regulatory subunit (p85 α)	↑	35	33	S49, WEHI, EoL
Hs.512712	Tubulin β polypeptide	↓	30	33	WEHI, Jurkat, CEM
M99054	Acid phosphatase type 5	↑		33, 32[h]	S49,WEHI, thymocytes
Hs.131924	G protein-coupled receptor 65	↑		33, 32[h]	S49, WEHI, thymocytes
D50683	TGF-β II Receptor α	↑	34,30		MM.1S, Jurkat, CEM
Hs.315562	Glutamate–cysteine ligase, modifier subunit	↑&↓	30,31		Pre B, Jurkat LS7, CEM
Hs.435051	CDK inhibitor 2D (p19, inhibits CDK4)	↑&↓	30,37		Jurkat, CEM, preB
Hs.118183	Hypothetical protein FLJ22833	↓		33, 32[h]	WEHI, S49, thymus
Hs.282326	Down syndrome critical region gene 1	↑	31,29,36[g],37		PreB, CEM
Hs.73958	Recombination-activating gene 1 (RAG 1)	↑&↓	31,29,36[g],37		PreB, CEM
Hs.202453	c-myc	↓	30,29,36[g]		Jurkat, CEM
Hs.443057	CD53 antigen	↑	31,29,36[g]		PreB, CEM
Hs.75462	BTG family, member 2	↑	31,36[g],37		PreB, CEM,
Hs.42322	Paralemmin 2	↑	31,36[g],37		PreB, CEM
Hs.528404	Integrin α 4 (antigen CD49D)	↓	31,29,36[g]		PreB, CEM
L19314	Human HRY gene, complete cds	↓	30,29,36[g]		Jurkat, CEM

[a]Genes are listed according to the number of systems wherein regulation was observed [b]Unigene number (starting with Hs.) or GeneBank accession number (all others) [c]Commonly used gene name [d]↑ and ↓ denote two-fold or greater gene induction or repression, respectively [e]References to human or mouse work, respectively [f]Cellular systems: *Human*: CEM, various subclones of the CCRF-CEM T-ALL cell line as specified in the respective publications; PreB, PreB-697 B-ALL cells; MM; multiple myeloma cell line MM1s; Jurkat; T-ALL cell lines stably transfected with either rat GR^{wt} or rat GR^{LS7} *Mouse*: WEHI, WEHI7.2 lymphoma cell line; S49, S49.A2 lymphoma cell line; thymus-1, normal C56BL/6 thymocytes; thymus-2, 18d fetal thymocytes from C57BL/6 wild-type mice or GR2KO mice [g]Reference Webb *et al*.[36] contains only genes regulated in CEM cells sensitive to GC-induced apoptosis but not those regulated both in GC-sensitive and GC-resistant cells. Thus, such genes may be particularly relevant for cell death induction [h]Although 59 genes were regulated, only seven were reported that were found to be regulated in S49 and WEHI as well[32] [i]Although many more genes were regulated, only 20 genes were reported in Mittelstadt and Ashwell[38], that is, those most strongly regulated in both mouse strains. Since GR2KO mice are resistant to GC-induced apoptosis, regulation of these genes may not suffice for cell death induction

possibilities or hypotheses. The list might, however, constitute the most informative collection of genes to date with strong evidence for regulation by GC in cells prone to GC-induced apoptosis. Regarding their function, these genes might be tentatively grouped into three classes: (i) genes directly implicated in death and survival decisions; (ii) genes whose (de)regulation might lead to cellular distress (thereby entailing apoptotic or (apo)necrotic cell death, as discussed in Figure 2 and (iii) genes not causal in the death response. The latter comprise three functionally distinct subgroups: genes that may counteract the apoptotic response (e.g., receptors for TGFβ or IL-7), others that may control clinically relevant GC effects such as cell cycle progression, and 'innocent bystanders'. Finally, regulation of the GR itself deserves separate mention, since its regulation determines extent and duration of all other regulatory responses. In the following, we discuss some of the evolving death pathways and current models. These pathways are not mutually exclusive; depending on the cellular context and other circumstances, either may be used preferentially or several may act in parallel in a single cell (Figure 3).

GC-induced apoptosis as result of direct regulation of death or survival genes

GC might directly activate the apoptotic machinery by regulating components of either the 'extrinsic' or 'intrinsic' pathways or both. Studies using the caspase 8 inhibitor *crmA* in transgenic mice[56] and human ALL cell lines[31,57] suggested that GC-induced apoptosis may not critically depend on the extrinsic pathway. However, in mouse thymocytes, FasL is induced by GC,[1,58] and Caspase-8 inhibition countered cytochrome *c* release and apoptosis.[59] Thus, depending on experimental circumstances, the extrinsic pathway may or may not contribute to GC-mediated death signaling. Evidence for involvement of the intrinsic pathway, particularly of members of the Bcl-2 family, has been provided in essentially all systems: GC apoptosis in thymocytes from APAF-1-[60,61] and caspase 9-[62,63] deficient mice is compromised (although not absent), and thymocytes from double knockout mice lacking the BH3-only molecules Bax and Bak[64] are GC resistant. Moreover, the single 'knockouts' of the BH3-only proteins Bim,[65] and Puma or Noxa[66] show partial GC resistance (Bax,[67] and Bid[68] knockouts cause mild, if any,

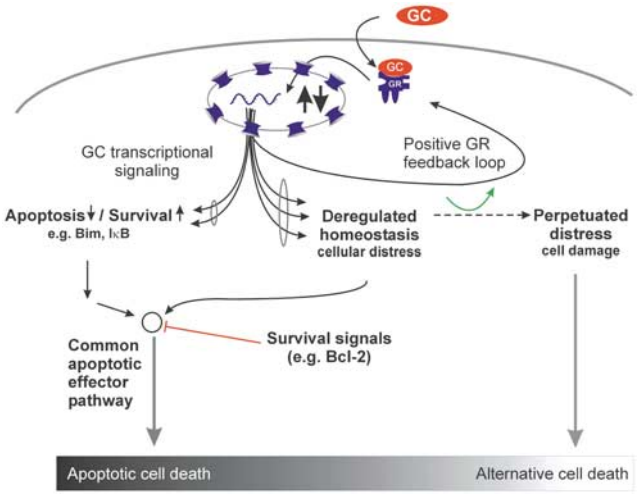

Figure 3 Proposed model for GC-induced apoptosis. GC may induce apoptosis by directly regulating typical apoptosis or survival genes, such as Bim or IkB (left side of the figure), or by inducing cellular distress that triggers the apoptotic cascade. In the presence of overexpression of antiapoptotic genes (such as Bcl-2), this mechanism may be blocked. If, under these circumstances, the GC-induced cellular distress is perpetuated by GR autoinduction, it may lead to (apo)necrotic cell death (right side of figure) (for further details see text)

deficiency in this response). Furthermore, overexpression of antiapoptotic Bcl-2 family members attenuated GC-induced cell death both in mouse thymocytes[69] and human ALL[47,70] and myeloma[71] cell lines. Since induction of proapoptotic[33,72] and repression of antiapoptotic[34,73] Bcl-2 family proteins has been observed in GC-treated cells, transcriptional deregulation of the Bcl-2 rheostat may be an essential principle for GC-induced apoptosis in many systems. However, as discussed below and depicted in Figure 3, the situation may become more complex in the presence of overexpressed antiapoptotic Bcl-2 family members or other prosurvival genes.

In addition to the regulation of components of the apoptotic machinery proper, GC may induce cell death by interfering with critical survival pathways. Perhaps the most intensively studied system is multiple myeloma, where interference with survival signaling through activation of the related adhesion focal tyrosine kinase (RAFTK; also known as Pyk2), a member of the focal adhesion kinase (FAK) subfamily, has been implicated in GC-induced apoptosis.[74] In support, IL-6 protected such cells from GC-induced apoptosis, and this was associated with RAFTK/Pyk2 inactivation mediated by the protein-tyrosine phosphatase SHP2.[75] However, GC regulation of RAFTK/Pyk2 was not observed in expression profiling studies of multiple systems (Table 1), suggesting that this mechanism might be specific for myeloma cells. Other examples for possible GC effects on survival pathways supported by the studies summarized in Table 1 include the induction of the NFkB inhibitor IkB[76] and of GILZ, which interacts with, and inactivates, NFkB[77] and AP-1.[78] Related DNA-binding-independent mechanisms (hence not readily detected by mRNA expression profiling studies) include direct protein–protein interaction of the GR with components of NFkB, AP-1 and other transcription factors like p53 implicated in death/survival decisions (reviewed in Geley *et al*.[27] and Herrlich[79]). Alternatively, or in addition, the above GR protein–protein interactions might account for the anti-inflammatory GC effects.[80]

Cell death as result of GC-induced cellular distress

Alternatively, or in addition to the above mechanisms, GC might induce apoptosis indirectly by gene (de)regulations that entail distress and cellular damage. This category might include the regulation of genes affecting metabolic pathways,[13,29] general transcription and/or translation,[30] production of, or response to, oxygen radicals,[81,82] Ca^{2+} fluxes[81,83] or intracellular pH[84] and volume control.[85] Examples from Table 1 that support these conclusions include the induction of the thioredoxin inhibitor, TXNIP, or repression of the lactate transporter, MCT-1. The former might contribute to increased oxidative stress, the latter to metabolic alterations, pH changes and/or disturbed volume control. As discussed in the Introduction, the resulting cellular distress may consecutively activate the apoptotic machinery. If apoptosis is blocked, for example, by overexpression of antiapoptotic Bcl-2 proteins or activated survival pathways, the cellular distress may become incompatible with cell survival and, if maintained for a sufficient time, lead to necrotic cell death. In support of this mechanism, high levels of transgenic Bcl-2 and Bcl-XL even in combination with saturating amounts of the pan-caspase inhibitor z-VAD failed to restore viability in GC-treated CCRF-CEM T-ALL cells in continuous presence of the drug. The resulting cell death was, however, delayed and showed altered morphology, including reduced DNA fragmentation and increased membrane permeability for vital dyes, as shown by time-lapse video microscopy (C Ploner, in preparation). Thus, there may be a continuous transition between two cell death forms, one rapid and showing typical apoptotic features in cells with low levels of Bcl-2 (and/or other antiapoptotic molecules) and another retarded form with more necrotic characteristics in cells with high levels of such proteins (Figure 3). The latter, slow cell death form may be critically dependent upon GR autoinduction (or at least lack of GR downregulation).

Mechanisms of resistance to GC-induced apoptosis

In general terms, GC resistance is defined as the inability of an individual cell or an entire organism to respond to all or a restricted number of GC responses. It can be absolute, as is the case in the absence of the GR, or relative and dependent on specific circumstances such as GC concentration, presence of apoptosis-inhibiting or -facilitating factors, etc. In the context of this review, GC resistance refers to the failure of lymphoid lineage cells to undergo GC-induced cell death under specific experimental or clinical conditions. Whether it affects other responses as well, whether it is context dependent or absolute, and what the underlying molecular mechanisms are, have considerable clinical consequences. For instance, if caused by GR gene mutations, GC resistance is absolute and irreversible, rendering the continuation of GC treatment with all its long-term side effects questionable. If

caused by regulatory mechanisms, therapeutic reversal of GC resistance might become an option.

An almost endless number of possible molecular mechanisms for GC resistance can be envisaged along the signal transduction pathways triggered by GC (Figure 4), and some of these mechanisms have recently been reviewed elsewhere.[5,8,9,52,86]) Conceptually, they may be grouped into 'upstream' and 'downstream' mechanisms. The former concern the GR, its ligand and GR-associated proteins that control its function, and have the potential to affect most, if not all, GC effects while the latter interfere with, and affect, only individual GC responses. In lymphoid malignancies, clinically relevant GC resistance means continuous expansion of tumor cells in the presence of GC, requiring resistance to both apoptosis induction and GC-mediated cell cycle arrest, processes that, at least in ALL cell lines, follow distinct pathways.[6] However, to simultaneously interfere with multiple pathways via 'downstream mechanisms' is considerably more complex than through 'upstream mechanisms'. Indeed, convincing evidence for a causative role in resistance to GC-induced apoptosis has so far mainly been provided for 'upstream mechanisms'.

'Upstream mechanisms-1': insufficient ligand

Most apical in the response is the requirement for sufficient intracellular levels of biologically active GC. This parameter is technically difficult to assess, but GC-like bioactivity can be determined in the plasma of patients during therapy.[87] Insufficient plasma levels may result from impaired uptake, increased steroid-binding proteins in the circulation or reduced converting enzyme activity, if prodrugs like prednisone are used. Intracellular GC levels may be reduced by overexpression of members of the large ABC transporter family, most notably the *mdr-1* gene- encoded P-glycoprotein

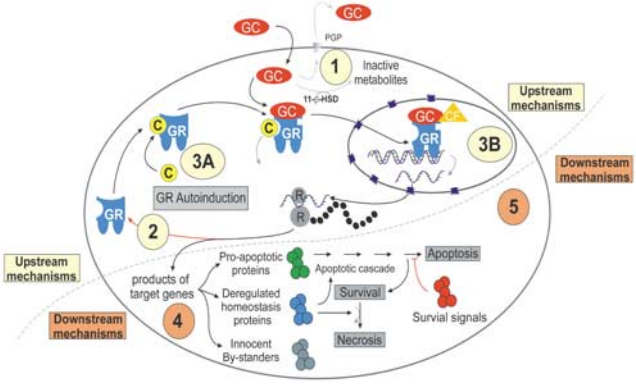

Figure 4 Principal mechanisms of GC resistance. Possible resistance mechanisms were organized along the GC signaling pathway and numbered consecutively: 'Upstream' mechanisms 1 and 2 have been detailed in the text, upstream mechanism 3 concerns deficiencies in GR-associated proteins in the cytoplasm (3A) and nucleus (3B), respectively (discussed in Kofler *et al*.[52]). 'Downstream' mechanisms encompass (4) defects in components of the specific response pathway or (5) crosstalk from other signaling pathways that interfere with and antagonize the death response. Abbreviations: 11β-HSD, 11β-hydroxysteroid dehydrogenase type 2; C, chaperones; CF, transcription cofactors, GC, glucocorticoid; GR, glucocorticoid receptor; Pgp, P-glycoprotein; R, ribosome

and the multidrug resistance-associated protein, MRP, as well as the lung-resistance protein (LRP), a major vault protein that formally does not belong to the ABC family but is still implicated in drug resistance (reviewed in Gottesman *et al*.[88]). In addition to affecting apoptotic responses to other agents as well, this form of GC resistance is characterized by its sensitivity to Pgp inhibitors, like verapramil or cyclosporin A, and its differing efficiency towards various GC analogues,[89] which might open therapeutic possibilities. *Mdr-1* gene overexpression has been made responsible for GC resistance in a mouse thymoma line,[90] but what role this form of GC resistance might play in patients is not clear.[8,88,91] Finally, GC resistance might be caused by expression of GC-metabolizing enzymes such as 11β-hydroxysteroid dehydrogenase type 2 that converts cortisol into inactive cortisone, as has been shown in rat osteosarcoma cells,[92] or mouse osteoblasts/osteocytes[14] transgenic for this enzyme.

'Upstream mechanisms-2': GR mutations, splice variants or insufficient expression

The next checkpoint in the pathway is the GR itself where mutations, occurrence of GR variants and insufficient expression might cause resistance. Numerous loss-of-function mutations in the GR gene have been observed in GC-resistant human ALL cell lines (e.g., Hala *et al*.[42] and Strasser-Wozak *et al*.[93]), but whether GR mutations constitute a major resistance mechanism *in vivo* remains unresolved. The combination of GC and chemotherapy, with its mutagenic potential, might indeed favor the development of, and subsequent selection for, GR mutations. However, one study found no evidence of mutations in the DNA- and ligand-binding domains of the GR in 22 chronic lymphatic leukemia patients subjected to combination chemotherapy,[94] and another study with $\sim$50 children with relapsed ALL provided only limited evidence for GR mutations as the cause of GC resistance (J Irving *et al*., submitted for publication).

GC resistance may also be caused by increased expression of GR variants (Figure 1) resulting from alternative splicing, polyadenylation or translational initiation, namely GR-β, GRγ, GR-P/GR-δ, GR-A and GR-B (for citations to the original literature see Tissing *et al*.[8] and Kofler *et al*.[52]). GR-P/GR-δ and GR-A were detected in a GC-resistant myeloma cell line, and GR-P in a number of hematopoietic and other malignancies as well as in normal lymphocytes, but how these variants might affect GC sensitivity remains controversial.[95,96] The GR-β splice variant reportedly encoded a dominant negative GR protein[97,98] and has been implicated in various forms of GC resistance, including patients with lymphoblastic malignancies.[99,100] However, there is little, if any, GRβ expression in various hematopoietic tumors, which makes its role in resistance development questionable.[50,96,101] Indeed, Haarman *et al*.[102] concluded that GRβ is not involved in GC resistance in childhood leukemia, although a possible involvement of GRγ in certain childhood leukemia subgroups could not be excluded. Whether GR-B affects sensitivity to GC-induced apoptosis in lymphoid malignancies is unknown.

GRα is the major functional GR isoform and, as discussed above, its expression is a critical factor for GC sensitivity in

numerous experimental systems. In clinical studies, GR expression levels above ~10 000 copies per cell at diagnosis correlated with beneficial outcome in childhood ALL,[103,104] but this correlation is not a consistent finding.[105,106] As discussed previously, GR levels at the onset of treatment may not be as important as GR expression kinetics (up- or downregulation) during treatment, but clinical studies addressing this question have not been reported thus far.

'Downstream mechanisms' interfering with death or activating survival signals

Theoretically, resistance to GC-induced apoptosis might result from unresponsiveness of, or mutations in, GC-regulated genes critical for death induction or from activation of genes and/or pathways interfering with the GC-induced death pathway (Figure 4). There are many reports in the literature on GC resistance by downstream mechanisms in experimental systems; however, in most, if not all, cases, the observed phenotype might be better referred to as reduced sensitivity rather than true long-term resistance with maintained clonogenic survival in the continuous presence of the drug. In patients, glutathione and glutathione *S*-transferase expression (reviewed in Tissing *et al*.[8] and Haarman *et al*.[86]) and alterations in the 'Bcl-2 rheostat' have attracted considerable attention. Regarding the latter, expression of Bcl-2 family members was investigated in numerous studies with somewhat conflicting outcomes. For instance, some investigators suggested that Bcl-XL[73] or the Bax-α:Bcl-2 ratio[107] might play a role in the protection of leukemic cells from GC-induced apoptosis, but one report found no alterations in Bax and Bcl-2 expression during *in vivo* chemotherapy,[108] and another concluded that neither Bcl-2 nor Bcl-XL, Mcl-1, Bax, Bad or Bak had prognostic significance in such children at diagnosis.[109] Interestingly, Bcl-2 was increased in relapsed ALL samples,[110] and downregulation of Bcl-2 or Bcl-XL by antisense oligonucleotides lead to sensitization of leukemia or myeloma cell lines, and freshly isolated myeloma cells from patients.[111,112]

Clinical significance and future perspectives

GC-induced apoptosis: therapeutic principle or surrogate marker?

In vitro and *in vivo* GC sensitivity are major prognostic factors in childhood ALL (reviewed in Tissing *et al*.[8] and Haarman *et al*.[86]). Children who respond well to an initial 8d monotherapy with prednisone in the BMF protocol have an excellent prognosis, whereas those who do not generally have an unfavorable outcome.[113] This correlation holds true for subgroups with poor outcome as well (infant ALL,[114] T-ALL,[115] Philadelphia chromosome-positive ALL[116]), that is, children with good prednisone *in vivo* response fare better than those with poor responses. In spite of this suggestive evidence, the crucial clinical question remains as to whether the cytolytic (and cytostatic) GC effect is, indeed, of additional therapeutic value or, alternatively, whether GC sensitivity simply defines a clinical entity that is particularly sensitive to conventional combination chemotherapy. In the former case, it is important to identify causes for GC resistance and develop improved therapy protocols that prevent and/or circumvent it. In the latter, GC should only be used for prognostic purposes but, because of its long-term side effects, might be withdrawn from therapy protocols.

Ethical reasons preclude clinical studies comparing protocols with and without GC to conclusively resolve this question. However, the deferral of GC from the initial month of induction therapy to the second month resulted in decreased event-free survival, and different types of GC (dexamethasone *versus* prednisolone) in induction and maintenance also influenced event-free survival (reviewed in Gayon and Carrel[4]). This clinical evidence, and the fact that GC provide an additional tool in the chemotherapeutic array, strongly argues for a critical therapeutic role of these steroids. Compared to other antileukemic drugs, GC have almost no acute side effects, lack cancerogenic activity and induce apoptosis that is relatively cell specific and independent of p53,[117,118] which is frequently mutated in hematopoietic malignancies. In spite of these advantages, interest in further investigating the therapeutic potential of GC is surprisingly low, perhaps because their antileukemic effect was discovered 50 years ago. Had this discovery been made in the era of Cleevec, interest in these compounds would probably be tremendous.

From a molecular understanding to the bedside: optimizing therapy protocols

Although patients with lymphoid malignancies may be treated successfully with existing protocols, there are many who are not, and even those who are cured suffer from considerable treatment-associated side effects, including the risk of secondary malignancies developing decades later, a threat particularly relevant in childhood ALL. There are numerous considerations centering around improving efficiency, reducing side effects and, most importantly, preventing or reverting GC resistance that may be present at the onset of treatment or develop during therapy (primary and secondary resistance, respectively). Current therapy protocols based on trial and error of a limited number of substances and combinations thereof are unlikely to represent optimal therapeutic regimens. Many compounds have been identified that potentiate the antileukemic GC effects, including histone deacetylase inhibitors,[119] immunophilin-targeting drugs,[120] immunomodulatory derivatives of thalidomide (IMiDs),[121] proteasome inhibitors such as PS-341,[122] and the anti-CD20 antibody rituximab.[123] These substances as well as new GC analogues with distinct pharmakokinetic properties (blood/brain or blood/testis barrier penetration; sensitivity to P-glycoprotein; etc.) might be combined in wide variety of ways with existing or emerging chemotherapeutics and drugs that target specific oncogenic pathways (for a review, see Anderson[124]). This complexity is further potentiated by an increasing number of entities among lymphoid malignancies, as defined by their expression profiles and polymorphic patient drug responses. Optimal protocols will need to be tailored to specific tumor subgroups and individual patients

('individualized medicine'). A profound molecular understanding combined with improved preclinical test models will be required to distill the almost infinite number of conceivable protocols to a few that can be subjected to clinical studies.

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References

1. Ashwell JD, Lu FW and Vacchio MS (2000) Glucocorticoids in T cell development and function. Annu. Rev. Immunol. 18: 309–345
2. Cidlowski JA, King KL, Evans-Storms RB, Montague JW, Bortner CD and Hughes Jr FM (1996) The biochemistry and molecular biology of glucocorticoid-induced apoptosis in the immune system. Recent Prog. Horm. Res. 51: 457–490
3. Jondal M, Pazirandeh A and Okret S (2001) A role for glucocorticoids in the thymus? Trends Immunol. 22: 185–186
4. Gaynon PS and Carrel AL (1999) Glucocorticosteroid therapy in childhood acute lymphoblastic leukemia. Adv. Exp. Med. Biol. 457: 593–605
5. Renner K, Ausserlechner MJ and Kofler R (2003) A conceptual view on glucocorticoid-induced apoptosis, cell cycle arrest and glucocorticoid resistance in lymphoblastic leukemia. Curr. Mol. Med. 3: 707–717
6. Ausserlechner MJ, Obexer P, Böck G, Geley S and Kofler R (2004) Cyclin D3 and c-myc control glucocorticoid-induced cell cycle arrest but not apoptosis in lymphoblastic leukemia cells. Cell Death Differ. 11: 165–174
7. McKay LI and Cidlowski JA (1999) Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. Endocr. Rev. 20: 435–459
8. Tissing WJ, Meijerink JP, den Boer ML and Pieters R (2003) Molecular determinants of glucocorticoid sensitivity and resistance in acute lymphoblastic leukemia. Leukemia 17: 17–25
9. Schaaf MJ and Cidlowski JA (2002) Molecular mechanisms of glucocorticoid action and resistance. J. Steroid Biochem. Mol. Biol. 83: 37–48
10. Greenstein S, Ghias K, Krett NL and Rosen ST (2002) Mechanisms of glucocorticoid-mediated apoptosis in hematological malignancies. Clin. Cancer Res. 8: 1681–1694
11. Distelhorst CW (2002) Recent insights into the mechanism of glucocorticosteroid-induced apoptosis. Cell Death Differ. 9: 6–19
12. Planey SL and Litwack G (2000) Glucocorticoid-induced apoptosis in lymphocytes. Biochem. Biophys. Res. Commun. 279: 307–312
13. Kofler R (2000) The molecular basis of glucocorticoid-induced apoptosis of lymphoblastic leukemia cells. Histochem. Cell Biol. 114: 1–7
14. O'Brien CA, Jia D, Plotkin LI, Bellido T, Powers CC, Stewart SA, Manolagas SC and Weinstein RS (2004) Glucocorticoids act directly on osteoblasts and osteocytes to induce their apoptosis and reduce bone formation and strength. Endocrinology 145: 1835–1841
15. Sapolsky RM, Romero LM and Munck AU (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21: 55–89
16. Druilhe A, Letuve S and Pretolani M (2003) Glucocorticoid-induced apoptosis in human eosinophils: mechanisms of action. Apoptosis 8: 481–495
17. Hammer S, Sauer B, Spika I, Schraut C, Kleuser B and Schafer-Korting M (2004) Glucocorticoids mediate differential anti-apoptotic effects in human fibroblasts and keratinocytes via sphingosine-1-phosphate formation. J. Cell Biochem. 91: 840–851
18. Yamaguchi S, Ohsaki Y, Nishigaki Y, Toyoshima E and Kikuchi K. (1999) Inhibition of human small cell lung cancer cell growth by methylprednisolone. Int. J. Oncol. 15: 1185–1190
19. Wessely O, Deiner EM, Beug H and Von Lindern M (1997) The glucocorticoid receptor is a key regulator of the decision between self-renewal and differentiation in erythroid progenitors. EMBO J. 16: 267–280
20. Stankova J, Turcotte S, Harris J and Rola-Pleszczynski M (2002) Modulation of leukotriene b(4) receptor-1 expression by dexamethasone: potential mechanism for enhanced neutrophil survival. J. Immunol. 168: 3570–3576
21. Amsterdam A and Sasson R (2002) The anti-inflammatory action of glucocorticoids is mediated by cell type specific regulation of apoptosis. Mol. Cell. Endocrinol. 189: 1–9
22. Fan W, Sui M and Huang Y (2004) Glucocorticoids selectively inhibit paclitaxel-induced apoptosis: mechanisms and its clinical impact. Curr. Med. Chem. 11: 403–411
23. Bloom JW, Chacko J, Lohman IC, Halonen M, Martinez FD and Miesfeld RL (2004) Differential control of eosinophil survival by glucocorticoids. Apoptosis 9: 97–104
24. Buttgereit F and Scheffold A (2002) Rapid glucocorticoid effects on immune cells. Steroids 67: 529–534
25. Laudet V and Gronemeyer H (2002) The Nuclear Receptor Facts Book. (London: Academic Press) pp. 1–462
26. Pratt WB and Toft DO (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr. Rev. 18: 306–360
27. Geley S, Fiegl M, Hartmann BL and Kofler R (1996) Genes mediating glucocorticoid effects and mechanisms of their regulation. Rev. Physiol. Biochem. Pharmacol. 128: 1–97
28. Maurer M, Trajanoski Z, Frey G, Hiroi N, Galon J, Willenberg HS, Gold PW, Chrousos GP, Scherbaum WA and Bornstein SR (2001) Differential gene expression profile of glucocorticoids, testosterone, and dehydroepiandrosterone in human cells. Horm. Metab Res. 33: 691–695
29. Tonko M, Ausserlechner MJ, Bernhard D, Helmberg A and Kofler R (2001) Gene expression profiles of proliferating *versus* G1/G0 arrested human leukemia cells suggest a mechanism for glucocorticoid-induced apoptosis. FASEB J. 15: 693–699
30. Obexer P, Certa U, Kofler R and Helmberg A (2001) Expression profiling of glucocorticoid-treated T-ALL cell lines: rapid repression of multiple genes involved in RNA-, protein- and nucleotide synthesis. Oncogene 20: 4324–4336
31. Planey SL, Abrams MT, Robertson NM and Litwack G (2003) Role of apical caspases and glucocorticoid-regulated genes in glucocorticoid-induced apoptosis of pre-B leukemic cells. Cancer Res. 63: 172–178
32. Wang Z, Malone MH, Thomenius MJ, Zhong F, Xu F and Distelhorst CW (2003) Dexamethasone-induced gene 2 (dig2) is a novel pro-survival stress gene induced rapidly by diverse apoptotic signals. J. Biol. Chem. 278: 27053–27058
33. Wang Z, Malone MH, He H, McColl KS and Distelhorst CW (2003) Microarray analysis uncovers the induction of the pro-apoptotic BH3-only protein Bim in multiple models of glucocorticoid induced apoptosis. J. Biol. Chem. 278: 23861–23867
34. Chauhan D, Auclair D, Robinson EK, Hideshima T, Li G, Podar K, Gupta D, Richardson P, Schlossman RL, Krett N, Chen LB, Munshi NC and Anderson KC (2002) Identification of genes regulated by dexamethasone in multiple myeloma cells using oligonucleotide arrays. Oncogene 21: 1346–1358
35. Chauhan S, Leach CH, Kunz S, Bloom JW and Miesfeld RL (2003) Glucocorticoid regulation of human eosinophil gene expression. J. Steroid Biochem. Mol. Biol. 84: 441–452
36. Webb MS, Miller AL, Johnson BH, Fofanov Y, Li T, Wood TG and Thompson EB (2003) Gene networks in glucocorticoid-evoked apoptosis of leukemic cells. J. Steroid Biochem. Mol. Biol. 85: 183–193
37. Yoshida NL, Miyashita T, U M, Yamada M, Reed JC, Sugita Y and Oshida T (2002) Analysis of gene expression patterns during glucocorticoid-induced apoptosis using oligonucleotide arrays. Biochem. Biophys. Res. Commun. 293: 1254–1261
38. Mittelstadt PR and Ashwell JD (2003) Disruption of glucocorticoid receptor exon 2 yields a ligand-responsive C-terminal fragment that regulates gene expression. Mol. Endocrinol. 17: 1534–1542

39. Valverde MA and Parker MG (2002) Classical and novel steroid actions: a unified but complex view. Trends Biochem. Sci. 27: 172–173
40. Gametchu B and Watson CS (2002) Correlation of membrane glucocorticoid receptor levels with glucocorticoid-induced apoptotic competence using mutant leukemic and lymphoma cells lines. J. Cell Biochem. 87: 133–146
41. Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R, Gass P, Schmid W, Herrlich P, Angel P and Schütz G (1998) DNA binding of the glucocorticoid receptor is not essential for survival. Cell 93: 531–541
42. Hala M, Hartmann BL, Böck G, Geley S and Kofler R (1996) Glucocorticoid receptor gene defects and resistance to glucocorticoid-induced apoptosis in human leukemic cell lines. Int. J. Cancer 68: 663–668
43. Geley S, Hartmann BL, Hala M, Strasser-Wozak EMC, Kapelari K and Kofler R (1996) Resistance to glucocorticoid-induced apoptosis in human T-cell acute lymphoblastic leukemia CEM-C1 cells is due to insufficient glucocorticoid receptor expression. Cancer Res. 56: 5033–5038
44. Helmberg A, Auphan N, Caelles C and Karin M (1995) Glucocorticoid-induced apoptosis of human leukemic cells is caused by the repressive function of the glucocorticoid receptor. EMBO J. 14: 452–460
45. Reichardt HM, Umland T, Bauer A, Kretz O and Schutz G (2000) Mice with an increased glucocorticoid receptor gene dosage show enhanced resistance to stress and endotoxic shock. Mol. Cell. Biol. 20: 9009–9017
46. Pazirandeh A, Xue Y, Prestegaard T, Jondal M and Okret S (2002) Effects of altered glucocorticoid sensitivity in the T-cell lineage on thymocyte and T-cell homeostasis. FASEB J. 16: 727–729
47. Brunet CL, Gunby RH, Benson RSP, Hickman JA, Watson AJM and Brady G (1998) Commitment to cell death measured by loss of clonogenicity is separable from the appearance of apoptotic markers. Cell Death Differ. 5: 107–115
48. Wallace AD and Cidlowski JA (2001) Proteasome-mediated glucocorticoid receptor degradation restricts transcriptional signaling by glucocorticoids. J. Biol. Chem. 276: 42714–42721
49. Gomi M, Moriwaki K, Katagiri S, Kurata Y and Thompson EB (1990) Glucocorticoid effects on myeloma cells in culture: correlation of growth inhibition with induction of glucocorticoid receptor messenger RNA. Cancer Res. 50: 1873–1878
50. Pedersen KB and Vedeckis WV (2003) Quantification and glucocorticoid regulation of glucocorticoid receptor transcripts in two human leukemic cell lines. Biochemistry 42: 10978–10990
51. Ramdas J, Liu W and Harmon JM (1999) Glucocorticoid-induced cell death requires autoinduction of glucocorticoid receptor expression in human leukemic T cells. Cancer Res. 59: 1378–1385
52. Kofler R, Schmidt S, Kofler A and Ausserlechner MJ (2003) Resistance to glucocorticoid-induced apoptosis in lymphoblastic leukemia. J. Endocrinol. 178: 19–27
53. Riml S, Schmidt S, Ausserlechner MJ, Geley S and Kofler R (2004) Glucocorticoid receptor heterozygosity combined with lack of receptor auto-induction causes glucocorticoid resistance in Jurkat acute lymphoblastic leukemia cells. Cell Death Differ 11(S1): S65–S72
54. Dieken ES and Miesfeld RL (1992) Transcriptional transactivation functions localized to the glucocorticoid receptor N terminus are necessary for steroid induction of lymphocyte apoptosis. Mol. Cell. Biol. 12: 589–597
55. Thompson EB, Nazareth LV, Thulasi R, Ashraf J, Harbour D and Johnson BH (1992) Glucocorticoids in malignant lymphoid cells: gene regulation and the minimum receptor fragment for lysis. J. Steroid Biochem. Mol. Biol. 41: 273–282
56. Smith KGC, Strasser A and Vaux DL (1996) CrmA expression in T lymphocytes of transgenic mice inhibits CD95 (Fas/APO-1)-transduced apoptosis, but does not cause lymphadenopathy or autoimmune disease. EMBO J. 15: 5167–5176
57. Geley S, Hartmann BL, Kapelari K, Egle A, Villunger A, Heidacher D, Greil R, Auer B and Kofler R (1997) The interleukin 1b-converting enzyme inhibitor crmA prevents Apo1/fas- but not glucocorticoid-induced poly(ADP-ribose) polymerase cleavage and apoptosis in lymphoblastic leukemia cells. FEBS Lett. 402: 36–40
58. D'Adamio F, Zollo O, Moraca R, Ayroldi E, Bruscoli S, Bartoli A, Cannarile L, Migliorati G and Riccardi C (1997) A new dexamethasone-induced gene of the leucine zipper family protects T lymphocytes from TCR/CD3-activated cell death. Immunity 7: 803–812
59. Marchetti MC, Di Marco B, Cifone G, Migliorati G and Riccardi C (2003) Dexamethasone-induced apoptosis of thymocytes: role of glucocorticoid receptor-associated Src kinase and caspase-8 activation. Blood 101: 585–593
60. Cecconi F, Alvarez-Bolado G, Meyer BI, Roth KA and Gruss P (1998) Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. Cell 94: 727–737
61. Yoshida H, Kong YY, Yoshida R, Elia AJ, Hakem A, Hakem R, Penninger JM and Mak TW (1998) Apaf1 is required for mitochondrial pathways of apoptosis and brain development. Cell 94: 739–750
62. Hakem R, Hakem A, Duncan GS, Henderson JT, Woo M, Soengas MS, Elia A, De la Pompa JL, Kagi D, Khoo W, Potter J, Yoshida R, Kaufman SA, Lowe SW, Penninger JM and Mak TW (1998) Differential requirement for Caspase 9 in apoptotic pathways *in vivo*. Cell 94: 339–352
63. Kuida K, Haydar TF, Kuan CY, Gu Y, Taya C, Karasuyama H, Su MSS, Rakic P and Flavell RA (1998) Reduced apoptosis and cytochrome *c*-mediated caspase activation in mice lacking Caspase 9. Cell 94: 325–337
64. Rathmell JC, Lindsten T, Zong WX, Cinalli RM and Thompson CB (2002) Deficiency in Bak and Bax perturbs thymic selection and lymphoid homeostasis. Nat. Immunol. 3: 932–939
65. Bouillet P, Metcalf D, Huang DC, Tarlinton DM, Kay TW, Kontgen F, Adams JM and Strasser A (1999) Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science 286: 1735–1738
66. Villunger A, Michalak EM, Coultas L, Mullauer F, Bock G, Ausserlechner MJ, Adams JM and Strasser A (2003) p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. Science 302: 1036–1038
67. Knudson CM, Tung KSK, Tourtellotte WG, Brown GAJ and Korsmeyer SJ (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science 270: 96–99
68. Yin XM, Wang K, Gross A, Zhao Y, Zinkel S, Klocke B, Roth KA and Korsmeyer SJ (1999) Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature 400: 886–891
69. Grillot DA, Merino R and Nunez G (1995) Bcl-XL displays restricted distribution during T cell development and inhibits multiple forms of apoptosis but not clonal deletion in transgenic mice. J. Exp. Med. 182: 1973–1983
70. Hartmann BL, Geley S, Löffler M, Hattmannstorfer R, Strasser-Wozak EMC, Auer B and Kofler R (1999) Bcl-2 interferes with the execution phase, but not upstream events, in glucocorticoid-induced leukemia apoptosis. Oncogene 18: 713–719
71. Feinman R, Koury J, Thames M, Barlogie B, Epstein J and Siegel DS (1999) Role of NF-kappaB in the rescue of multiple myeloma cells from glucocorticoid-induced apoptosis by bcl-2. Blood 93: 3044–3052
72. Han J, Flemington C, Houghton AB, Gu Z, Zambetti GP, Lutz RJ, Zhu L and Chittenden T (2001) Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals. Proc. Natl. Acad. Sci. USA 98: 11318–11323
73. Casale F, Addeo R, D'Angelo V, Indolfi P, Poggi V, Morgera C, Crisci S and Di Tullio MT (2003) Determination of the *in vivo* effects of prednisone on Bcl-2 family protein expression in childhood acute lymphoblastic leukemia. Int. J. Oncol. 22: 123–128
74. Chauhan D, Hideshima T, Pandey P, Treon S, Teoh G, Raje N, Rosen S, Krett N, Husson H, Avraham S, Kharbanda S and Anderson KC (1999) RAFTK/PYK2-dependent and -independent apoptosis in multiple myeloma cells. Oncogene 18: 6733–6740
75. Chauhan D, Pandey P, Hideshima T, Treon S, Raje N, Davies FE, Shima Y, Tai YT, Rosen S, Avraham S, Kharbanda S and Anderson KC (2000) SHP2 mediates the protective effect of interleukin-6 against dexamethasone-induced apoptosis in multiple myeloma cells. J. Biol. Chem. 275: 27845–27850
76. Auphan N, DiDonato JA, Rosette C, Helmberg A and Karin M (1995) Immunosuppression by glucocorticoids: inhibition of NF-kappaB activity through induction of IkappaB synthesis. Science 270: 286–290
77. Ayroldi E, Migliorati G, Bruscoli S, Marchetti C, Zollo O, Cannarile L, D'Adamio F and Riccardi C (2001) Modulation of T-cell activation by the glucocorticoid-induced leucine zipper factor via inhibition of nuclear factor kappaB. Blood 98: 743–753
78. Mittelstadt PR and Ashwell JD (2001) Inhibition of AP-1 by the glucocorticoid-inducible protein GILZ. J. Biol. Chem. 276: 29603–29610

79. Herrlich P (2001) Cross-talk between glucocorticoid receptor and AP-1. Oncogene 20: 2465–2475
80. Caldenhoven E, Liden J, Wissink S, Van de Stolpe A, Raaijmakers J, Koenderman L, Okret S, Gustafsson J and Van der Saag PT (1995) Negative cross-talk between RelA and the glucocorticoid receptor: a possible mechanism for the antiinflammatory action of glucocorticoids. Mol. Endocrinol. 9: 401–412
81. Fernandez A, Kiefer J, Fosdick L and McConkey DJ (1995) Oxygen radical production and thiol depletion are required for Ca^{2+}-mediated endogenous endonuclease activation in apoptotic thymocytes. J. Immunol. 155: 5133–5139
82. Tome ME and Briehl MM (2001) Thymocytes selected for resistance to hydrogen peroxide show altered antioxidant enzyme profiles and resistance to dexamethasone-induced apoptosis. Cell Death Differ. 8: 953–961
83. Distelhorst CW and Dubyak G (1998) Role of calcium in glucocorticosteroid-induced apoptosis of thymocytes and lymphoma cells: resurrection of old theories by new findings. Blood 91: 731–734
84. Matsuyama S and Reed JC (2000) Mitochondria-dependent apoptosis and cellular pH regulation. Cell Death Differ. 7: 1155–1165
85. Gomez-Angelats M, Bortner CD and Cidlowski JA (2000) Cell volume regulation in immune cell apoptosis. Cell Tissue Res. 301: 33–42
86. Haarman EG, Kaspers GJ and Veerman AJ (2003) Glucocorticoid resistance in childhood leukaemia: mechanisms and modulation. Br. J. Haematol. 120: 919–929
87. Raivio T, Palvimo JJ, Kannisto S, Voutilainen R and Janne OA (2002) Transactivation assay for determination of glucocorticoid bioactivity in human serum. J. Clin. Endocrinol. Metab 87: 3740–3744
88. Gottesman MM, Fojo T and Bates SE (2002) Multidrug resistance in cancer: role of ATP-dependent transporters. Nat. Rev. Cancer 2: 48–58
89. Karssen AM, Meijer OC, van dS, I, Lucassen PJ, de Lange EC, de Boer AG and De Kloet ER (2001) Multidrug resistance P-glycoprotein hampers the access of cortisol but not of corticosterone to mouse and human brain. Endocrinology 142: 2686–2694
90. Bourgeois S, Gruol DJ, Newby RF and Rajah FM (1993) Expression of an *mdr* gene is associated with a new form of resistance to dexamethasone-induced apoptosis. Mol. Endocrinol. 7: 840–851
91. den Boer ML, Pieters R, Kazemier KM, Rottier MM, Zwaan CM, Kaspers GJ, Janka-Schaub G, Henze G, Creutzig U, Scheper RJ and Veerman AJ (1998) Relationship between major vault protein/lung resistance protein, multidrug resistance-associated protein, P-glycoprotein expression, and drug resistance in childhood leukemia. Blood 91: 2092–2098
92. Rabbitt EH, Lavery GG, Walker EA, Cooper MS, Stewart PM and Hewison M (2002) Prereceptor regulation of glucocorticoid action by 11beta-hydroxysteroid dehydrogenase: a novel determinant of cell proliferation. FASEB J. 16: 36–44
93. Strasser-Wozak EMC, Hattmannstorfer R, Hála M, Hartmann BL, Fiegl M, Geley S and Kofler R (1995) Splice site mutation in the glucocorticoid receptor gene causes resistance to glucocorticoid-induced apoptosis in a human acute leukemic cell line. Cancer Res. 55: 348–353
94. Soufi M, Kaiser U, Schneider A, Beato M and Westphal HM (1995) The DNA and steroid binding domains of the glucocorticoid receptor are not altered in mononuclear cells of treated CLL patients. Exp. Clin. Endocrinol. Diabetes 103: 175–183
95. Krett NL, Pillay S, Moalli PA, Greipp PR and Rosen ST (1995) A variant glucocorticoid receptor messenger RNA is expressed in multiple myeloma patients. Cancer Res. 55: 2727–2729
96. De Lange P, Segeren CM, Koper JW, Wiemer E, Sonneveld P, Brinkmann AO, White A, Brogan IJ, De Jong FH and Lamberts SW (2001) Expression in hematological malignancies of a glucocorticoid receptor splice variant that augments glucocorticoid receptor-mediated effects in transfected cells. Cancer Res. 61: 3937–3941
97. Oakley RH, Jewell CM, Yudt MR, Bofetiado DM and Cidlowski JA (1999) The dominant negative activity of the human glucocorticoid receptor beta isoform. Specificity and mechanisms of action. J. Biol. Chem. 274: 27857–27866
98. De Castro M, Elliot S, Kino T, Bamberger C, Karl M, Webster E and Chrousos GP (1996) The non-ligand binding beta-isoform of the human glucocorticoid receptor (hGR beta): tissue levels, mechanism of action, and potential physiologic role. Mol. Med. 2: 597–607
99. Webster JC, Oakley RH, Jewell CM and Cidlowski JA (2001) Proinflammatory cytokines regulate human glucocorticoid receptor gene expression and lead to the accumulation of the dominant negative beta isoform: a mechanism for the generation of glucocorticoid resistance. Proc. Natl. Acad. Sci. USA 98: 6865–6870
100. Longui CA, Vottero A, Adamson PC, Cole DE, Kino T, Monte O and Chrousos GP (2000) Low glucocorticoid receptor alpha/beta ratio in T-cell lymphoblastic leukemia. Horm. Metab. Res. 32: 401–406
101. Hecht K, Carlstedt-Duke J, Stierna P, Gustafsson JÅ, Brönnegård M and Wikström AC (1997) Evidence that the b-isoform of the human glucocorticoid receptor does not act as a physiologically significant repressor. J. Biol. Chem. 272: 26659–26664
102. Haarman EG, Kaspers GJ, Pieters R, Rottier MM and Veerman AJ (2004) Glucocorticoid receptor alpha, beta and gamma expression vs *in vitro* glucocorticod resistance in childhood leukemia. Leukemia 18: 530–537
103. Pui CH, Dahl GV, Rivera G, Murphy SB and Costlow ME (1984) The relationship of blast cell glucocorticoid receptor levels to response to single-agent steroid trial and remission response in children with acute lymphoblastic leukemia. Leuk. Res. 8: 579–585
104. Kato GJ, Quddus FF, Shuster JJ, Boyett J, Pullen JD, Borowitz MJ, Whitehead VM, Crist WM and Leventhal BG (1993) High glucocorticoid receptor content of leukemic blasts is a favorable prognostic factor in childhood acute lymphoblastic leukemia. Blood 82: 2304–2309
105. Lauten M, Cario G, Asgedom G, Welte K and Schrappe M (2003) Protein expression of glucocorticoid receptor in childhood acute lmphoblastic leukaemia. Haematologica 88: 1253–1258
106. Csoka M, Bocsi J, Falus A, Szalai C, Klujber V, Szende B and Schuler D (1997) Glucocorticoid-induced apoptosis and treatment sensitivity in acute lymphoblastic leukemia of children. Pediatr. Hematol. Oncol. 14: 433–442
107. Salomons GS, Brady HJM, Verwijs-Janssen M, Van den Berg JD, Hart AAM, Van den Berg H, Behrendt H, Hählen K and Smets LA (1997) The Baxa:Bcl-2 ratio modulates the response to dexamethasone in leukaemic cells and is highly variable in childhood acute leukaemia. Int. J. Cancer 71: 959–965
108. Stahnke K, Eckhoff S, Mohr A, Meyer LH and Debatin KM (2003) Apoptosis induction in peripheral leukemia cells by remission induction treatment *in vivo*: selective depletion and apoptosis in a CD34+ subpopulation of leukemia cells. Leukemia 17: 2130–2139
109. Salomons GS, Smets LA, Verwijs-Janssen M, Hart AA, Haarman EG, Kaspers GJ, Wering EV, Der Does-Van Den Berg AV and Kamps WA (1999) Bcl-2 family members in childhood acute lymphoblastic leukemia: relationships with features at presentation, *in vitro* and *in vivo* drug response and long-term clinical outcome. Leukemia 13: 1574–1580
110. Haarman EG, Kaspers GJ, Pieters R, Van Zantwijk CH, Broekema GJ, Hahlen K and Veerman AJ (1999) BCL-2 expression in childhood leukemia *versus* spontaneous apoptosis, drug induced apoptosis, and *in vitro* drug resistance. Adv. Exp. Med. Biol. 457: 325–333
111. Broome HE, Yu AL, Diccianni M, Camitta BM, Monia BP and Dean NM (2002) Inhibition of Bcl-xL expression sensitizes T-cell acute lymphoblastic leukemia cells to chemotherapeutic drugs. Leuk. Res. 26: 311–316
112. Liu Q and Gazitt Y (2003) Potentiation of dexamethasone, taxol and Ad-p53-induced apoptosis by Bcl-2 anti-sense oligodeoxynucleotides in drug-resistant multiple myeloma cells. Blood 101: 4105–4114
113. Reiter A, Schrappe M, Ludwig W-D, Hiddemann W, Sauter S, Henze G, Zimmermann M, Lampert F, Havers W, Niethammer D, Odenwald E, Ritter J, Mann G, Welte K, Gadner H and Riehm H (1994) Chemotherapy in 998 unselected childhood acute lymphoblastic leukemia patients. Results and conclusions of the multicenter trial ALL-BFM 86. Blood 84: 3122–3133
114. Dordelmann M, Reiter A, Borkhardt A, Ludwig WD, Gotz N, Viehmann S, Gadner H, Riehm H and Schrappe M (1999) Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia. Blood 94: 1209–1217
115. Arico M, Basso G, Mandelli F, Rizzari C, Colella R, Barisone E, Zanesco L, Rondelli R, Pession A and Masera G (1995) Good steroid response *in vivo* predicts a favorable outcome in children with T-cell acute lymphoblastic leukemia. The Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP). Cancer 75: 1684–1693
116. Schrappe M, Arico M, Harbott J, Biondi A, Zimmermann M, Conter V, Reiter A, Valsecchi MG, Gadner H, Basso G, Bartram CR, Lampert F, Riehm H and Masera G (1998) Philadelphia chromosome-positive (Ph+) childhood acute

lymphoblastic leukemia: good initial steroid response allows early prediction of a favorable treatment outcome. Blood 92: 2730–2741
117. Geley S, Hartmann BL, Hattmannstorfer R, Löffler M, Ausserlechner MJ, Bernhard D, Sgonc R, Strasser-Wozak EMC, Ebner M, Auer B and Kofler R (1997) P53-induced apoptosis in the human T-ALL cell line CCRF-CEM. Oncogene 15: 2429–2437
118. Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML and Wyllie AH (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature 362: 849–852
119. Buckley AR, Krumenacker JS, Buckley DJ, Leff MA, Magnuson NS, Reed JC, Miyashita T, De Jong G and Gout PW (1997) Butyrate-induced reversal of dexamethasone resistance in autonomous rat Nb2 lymphoma cells. Apoptosis 2: 518–528
120. Stromberg T, Dimberg A, Hammarberg A, Carlson K, Osterborg A, Nilsson K and Jernberg-Wiklund H (2004) Rapamycin sensitizes multiple myeloma cells to apoptosis induced by dexamethasone. Blood 103: 3138–3147
121. Hideshima T, Chauhan D, Shima Y, Raje N, Davies FE, Tai YT, Treon SP, Lin B, Schlossman RL, Richardson P, Muller G, Stirling DI and Anderson KC (2000) Thalidomide and its analogs overcome drug resistance of human multiple myeloma cells to conventional therapy. Blood 96: 2943–2950
122. Hideshima T, Richardson P, Chauhan D, Palombella VJ, Elliott PJ, Adams J and Anderson KC (2001) The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. Cancer Res. 61: 3071–3076
123. Rose AL, Smith BE and Maloney DG (2002) Glucocorticoids and rituximab *in vitro*: synergistic direct antiproliferative and apoptotic effects. Blood 100: 1765–1773
124. Anderson KC (2003) Moving disease biology from the lab to the clinic. Cancer 97: 796–801
125. Breslin MB, Geng CD and Vedeckis WV (2001) Multiple promoters exist in the human GR gene, one of which is activated by glucocorticoids. Mol. Endocrinol. 15: 1381–1395

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Client–Server environment for high-performance gene expression data analysis

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ABSTRACT

Summary: We have developed a platform independent, flexible and scalable Java environment for high-performance large-scale gene expression data analysis, which integrates various computational intensive hierarchical and non-hierarchical clustering algorithms. The environment includes a powerful client for data preparation and results visualization, an application server for computation and an additional administration tool. The package is available free of charge for academic and non-profit institutions.

Availability: http://genome.tugraz.at/Software

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INTRODUCTION

High-throughput gene expression analysis using oligonucleotide or cDNA microarrays is becoming increasingly important in many areas of basic and applied biomedical research. The microarray technology itself is developing rapidly, leading to an increasing density of the elements spotted onto a single slide. However, these genome-wide microarrays pose significant challenges on the data analysis tools. Many gene expression data mining algorithms utilize a similarity matrix as a starting point, in which the distances between all genes are calculated on the basis of a similarity function (Eisen *et al.*, 1998). The similarity matrix is a triangular matrix containing $(n^2 - n)/2$ elements, where n is the number of genes. Consequently, the similarity matrix of a genome-wide array with 30 000 genes requires almost 1.7 GB (2^{30} b) of RAM, assuming that each cell is represented by a floating point value of 4 B. Moreover, this is just one of many matrices, lists, and lookup tables mandatory for the calculation of a gene expression clustering or classification. It is noteworthy that the Java Virtual Machine on 32-bit computer architectures like Personal and Apple Computers is limited to 2 GB of memory. Thus, more demanding jobs using some of the popular cluster analysis tools (Sturn *et al.*, 2002) require costly 64-bit soft- and hardware architecture. Due to these constraints, data analysis of genomic scale microarrays becomes impractical or even impossible to perform on commonly used workstations. Computer architecture, CPU performance, amount of addressable and available memory, and costs are the limiting factors. Consequently, memory and calculation intensive tasks have to be outsourced to high-performance servers. We have therefore further developed our gene expression data analysis suite Genesis (Sturn *et al.*, 2002) to be capable of using the advantages of outsourcing the calculations to in-house or remote application servers.

PROGRAM OVERVIEW

The client–server environment (Fig. 1) consists of a versatile, platform independent, and easy to use Java client for data preprocessing and results visualization (Genesis Client), an application server (Genesis Server) for computation of Hierarchical Clustering (HCL; Eisen *et al.*, 1998), Self Organizing Maps (SOM; Tamayo *et al.*, 1999), k-means Clustering (KMC; Tavazoie *et al.*, 1999), and Support Vector Machines (SVM; Brown *et al.*, 2000), as well as an additional administration tool for statistics, job handling, and user management (Genesis Server Client). Data analysis is prepared in Genesis Client and the jobs are distributed to an available Genesis Server, where calculation is started and results are stored until they are fetched by the client. At all times the client is informed about status and progress of the calculation task. Nevertheless, all server jobs are completely independent from the client, so that the client may be turned off during calculation and restarted again later to retrieve the computed results. The user management system of the server warrants that only enrolled users have the rights to submit jobs and get their progress information and results. Additionally, it provides the functionality to specify the

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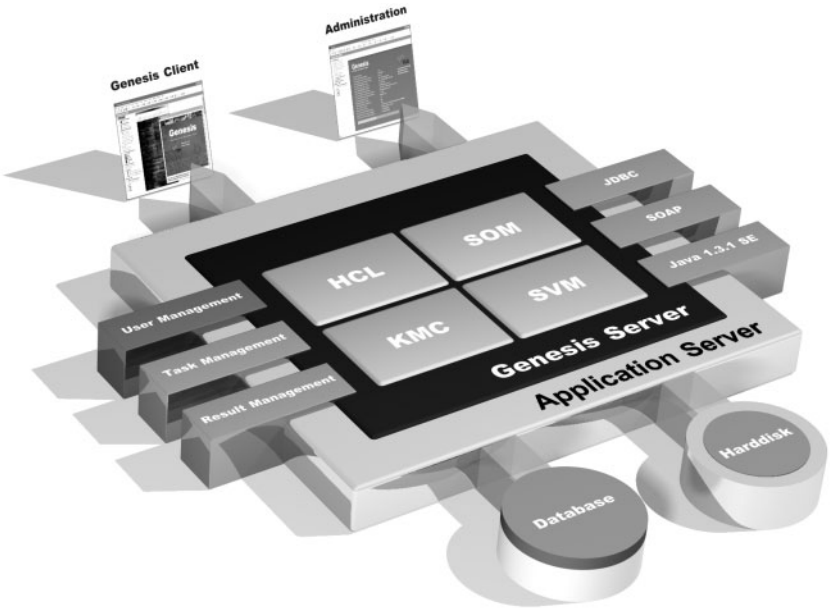

Fig. 1. Block diagram of the Genesis Server. The Genesis Server is executed on an application server and includes four data mining algorithms for large-scale gene expression data analysis: HCL (Hierarchical Clustering), SOM (Self-Organizing Maps), KMC (k-means Clustering), SVM (Support Vector Machine). Additionally, the server has a user and task management unit as well as a unit to handle, store and retrieve calculation results. The server is connected to a database for user and job information storage and uses a hard-disk to store the calculated results. Additional mandatory objects are the Java Runtime Environment 1.3.1 SE (standard edition) or later, SOAP (Simple Object Access Protocol) for communication between the clients and the server, and a JDBC (Java Database Connectivity) driver for the database connection.

number of calculation tasks each user is allowed to calculate simultaneously and in total. For controlling the server we have enclosed the standalone application Genesis Server Client, which enables system administrators to add or change user accounts in a straightforward manner, observe the server status, and abort specific calculation tasks if necessary. It also provides information on all calculated jobs by accessing the database incorporated into the Genesis Server. The latter is used to handle jobs, user accounts, and results in a reliable and secure environment. Our implementation uses the free available application server JBoss (http://www.jboss.org), is completely developed in Java, and available free of charge to academic and non-profit organizations. This renders it, to the best of our knowledge, the most cost effective solution for distributed high-performance gene expression data analysis. The Genesis Server environment is also scalable to high-performance multiprocessor servers. Up to date, the package has been tested on Windows 2000/XP, Linux (2 Intel PIII, 2 GB RAM), Solaris (Sun Fire V880, 4 UltraSPARC III, 8 GB RAM) and Tru64 Unix (AlphaServer ES45, 4 Alpha processors, 16 GB RAM) platforms.

FUTURE DEVELOPMENT

Present and future work will focus on porting the server to computer cluster environments to parallelize the huge computational tasks of gene expression clustering using bootstrapping and automatic parameter fitting. Additionally a job queuing system is in development to further improve performance and usability.

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REFERENCES

Brown,M.P., Grundy,W.N., Lin,D., Cristianini,N., Sugnet,C.W., Furey,T.S., Ares,Jr,M. and Haussler,D. (2000) Knowledge-based analysis of microarray gene expression data by using support vector machines. *Proc. Natl Acad. Sci. USA*, **97**, 262–267.

Eisen,M.B., Spellman,P.T., Brown,P.O. and Botstein,D. (1998) Cluster analysis and display of genome-wide expression patterns. *Proc. Natl Acad. Sci. USA*, **95**, 14863–14868.

Sturn,A., Quackenbush,J. and Trajanoski,Z. (2002) Genesis: cluster analysis of microarray data. *Bioinformatics*, **18**, 207–208.

Tamayo,P., Slonim,D., Mesirov,J., Zhu,Q., Kitareewan,S., Dmitrovsky,E., Lander,E.S. and Golub,T.R. (1999) Interpreting patterns of gene expression with self-organizing maps: methods and application to hematopoietic differentiation. *Proc. Natl Acad. Sci. USA*, **96**, 2907–2912.

Tavazoie,S., Hughes,J.D., Campbell,M.J., Cho,R.J. and Church,G.M. (1999) Systematic determination of genetic network architecture. *Nat. Genet.*, **22**, 281–285.