Gene Expression Profile of B16(F10) Murine Melanoma Cells Exposed to Hypoxic Conditions In Vitro

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Hypoxia is an important feature of tumor microenvironment, exerting far-reaching effects on cells and contributing to cancer progression. Previous studies have established substantial differences in hypoxia response between various cell lines. Investigating this phenomenon in melanoma cells contributes to a better understanding of cell lineage-specific hypoxia response and could point out novel hypoxia-regulated genes. We investigated transcriptional activity of B16(F10) murine melanoma cells cultured for 24 h under hypoxic (nominal 1% O₂, 15 samples including controls) and hypoxia-mimicking conditions (cobalt chloride, 100 or 200 μM, 6 samples including controls). Gene expression profiles were analyzed using MG-U74Av2 oligonucleotide microarrays. Data analysis revealed 2541 probesets (FDR <5%) for 1% oxygen experiment and 364 probesets (FDR <5%) for cobalt chloride, which showed differences in expression levels. Analysis of hypoxia-regulated genes (true hypoxia, 1% O₂) by stringent Family-Wise Error Rate estimation indicated 454 significantly changed transcripts ($p < 0.05$). The most upregulated genes were Lgals3, Selenbp1, Nppb (more than ten-fold increase). We observed significant differences in expression levels of genes regulating glycolysis (Pfkfb3, Hk2, Aldoa, Eno2), apoptosis (Bcl2, Bcl2l1, Bcl6, Bcl7a, Bcl7b, Bcl7c, Bcl7d, Bcl7e, Bcl7f, Bcl7g, Bcl7h, Bcl7i, Bcl7j, Bcl7k, Bcl7l, Bcl7m, Bcl7n, Bcl7o, Bcl7p, Bcl7q, Bcl7r, Bcl7s, Bcl7t, Bcl7u, Bcl7v, Bcl7w, Bcl7x, Bcl7y, Bcl7z), transcription (Bhlhe40, Sap30, Atf3, Mxi1), angiogenesis (Vegfa, Adm, Anxa2, Ctgf), adhesion (Pkp2, Itga4, Mcam), migration (Cnn2, Tmsb4x), and other processes. Both true hypoxia and hypoxia mimicry induced HIF-1-regulated genes. However, unsupervised analysis (Singular Value Decomposition) revealed distinct differences in gene expression between these two experimental conditions. Contrary to hypoxia, cobalt chloride caused suppression of gene expression rather than stimulation, especially concerning transcripts related to proliferation, immune response, DNA repair, and melanin biosynthesis.

Key words: Hypoxia; Cobalt chloride; Melanoma; Oligonucleotide microarrays;
Singular Value Decomposition

INTRODUCTION

Hypoxia is a common phenomenon associated with various solid tumors. It is caused by insufficient oxygen supply that results mainly from aberrant ar-

chitecture of tumor vessels. Cells respond to stressing conditions by turning on expression programs that enable them not only to adapt to such conditions and survive but also to proliferate or leave the adverse microenvironment (15). The “hypoxic phenotype” is

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mainly regulated by transcription factor HIF-1, which stimulates expression of proangiogenic factors (VEGF, ADM), apoptosis regulators (BNIP3), glycolytic enzymes (HK2, ALDO3, PFK), transport proteins (GLUT1, GLUT3), and others (35).

Hypoxia is in itself an obstacle to the treatment of solid tumors. Low oxygen tension decreases effectiveness of both radiation therapy and chemotherapy. By inducing molecular changes that promote a more malignant cell phenotype, hypoxia also leads to selection of apoptosis-insensitive cancer cells that possess higher metastatic potential. Such changes involve stimulation of genes encoding drug resistance as well as proangiogenic and antiapoptotic factors. Low oxygen tension also affects activity of DNA repair enzymes, adhesion molecules, and proteins involved in cell invasiveness (16,24). Although hypoxia exerts common biological effects, recent studies have nonetheless shown that there exist substantial differences in gene expression profiles among different cell lines (4,6). The knowledge concerning hypoxia-regulated cell-specific transcripts may be helpful both in deeper understanding of the hypoxia response mechanisms and also in seeking potential prognostic markers and therapeutic targets.

DNA microarray technology offers a convenient tool for identifying gene expression changes caused by various stimuli. Using this technology, the effects of induced hypoxia were studied in various models (4,6,11,43,47). Microarray analyses reveal hypoxia-regulated single genes as well as allow discerning sets of genes predominantly regulated by oxygen tension and specifying transcripts involved in particular hypoxia-dependent cellular processes.

Even though hypoxia was shown to enhance metastatic potential of B16(F10) murine melanoma cells *in vivo* (30), to our knowledge there have been no microarray technology-based reports for this cell line concerning transcriptional activity under hypoxia. Thus, the aim of our study was to thoroughly analyze hypoxia-induced gene expression changes in B16(F10) murine melanoma cells cultures using high-density microarrays and to search for hypoxia-regulated transcripts that might be specific for melanoma and/or associated with aggressiveness. We employed three methods of inducing hypoxia *in vitro*. Some cell cultures were exposed to decreased levels of oxygen, either in an O₂-regulated incubator or in a closed (static) chamber; these two conditions are herein referred to as "true hypoxia." The third method employed chemical hypoxia mimicry by means of cobalt chloride added to culture media; this has been known to interfere with oxygen-dependent cell signaling pathways [see, e.g. (34)].

MATERIALS AND METHODS

Cell Culture

B16(F10) melanoma cells obtained from ATCC (American Type Culture Collection) were cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (ICN) and maintained in a humidified incubator (37°C, 5% CO₂, 21% O₂). Upon reaching 70–80% confluence, the cell cultures were exposed to hypoxic conditions in the Billups-Rothenberg chamber (nominal 1% O₂, 5% CO₂, 94% N₂) or an oxygen flow-regulated (referred to as O₂-regulated) incubator (Jouan) with controlled gas flow (1% O₂, 5% CO₂, 94% N₂). The O₂-regulated incubator experiment was repeated twice. Alternatively, CoCl₂ (f.c. 100 or 200 µM) was added to cell culture medium. The exposure was maintained for 24 h. Each experiment was performed in triplicate (hypoxia samples) or duplicate (CoCl₂ and control samples).

Isolation of RNA From Cell Culture

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), followed by DNaseI (Qiagen) digestion and repurification (RNA clean-up protocol). The quantity of isolated RNA was estimated spectrophotometrically by measuring absorbance at 260 nm. RNA quality was assessed by 1.2% agarose gel electrophoresis.

cRNA Synthesis and Hybridization to Microarrays

All procedures were performed according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Total RNA (8 µg) from each sample was used as a template for cDNA and subsequent cRNA syntheses. Fragmented cRNA was hybridized to murine oligonucleotide GeneChip MG-U74Av2 microarrays (Affymetrix). Arrays were scanned by Hewlett-Packard GeneArray G2500A scanner with MAS5 software (Affymetrix).

Statistical Comparison of Gene Expression Levels

The preprocessing of data was carried out by Robust MultiArray Analysis (RMA) using the Bioconductor Affy package (8). Statistical comparisons were performed by GeneSpring 7.2 (Silicon Genetics, Redwood City, CA). Welch *t*-test was used and both Family-Wise Error Rate (Westfall-Young algorithm) and False Discovery Rate (Benjamini-Hochberg method) were calculated. An alternative method of gene selection, *limma* (38), based on moderated Bayesian *t*-statistic was applied, also providing False Discovery Rate estimation by Benjamini-Hochberg

method. Hierarchical clustering was done in Gene Spring software by centroid clustering referred to as the “average-linkage” method, with Pearson correlation around zero as the distance metric.

Singular Value Decomposition

Singular Value Decomposition (SVD)-based algorithm was applied as an unsupervised tool for analyzing the major sources of variability in the dataset. The algorithm of matrix decomposition was used to obtain orthogonal vectors called characteristic modes. Each mode represents major independent and uncorrelated variability patterns in the analyzed data. For the obtained mode profiles, genes that bore greatest similarity to each expression pattern were selected, as described previously (20,36). The whole procedure was carried out using MATLAB (MathWorks, Natick, MA) on the dataset preprocessed by RMA and column- and row-normalized (subtraction of mean and division by standard deviation).

Gene Ontology Analysis

Biological relevance of the obtained gene sets was analyzed by Gene Ontology classification. GOHyperG Bioconductor package (9) was used to select GO classes that were significantly overrepresented in the analyzed gene lists, based on hypergeometric distribution. As the obtained results were not corrected for multiple comparisons, only those with a *p*-value lower than 0.005 were interpreted as statistically significant.

Real-Time Quantitative PCR Validation of Microarray Data

The sequences for chosen murine genes were blasted against the murine genome and intron–exon boundaries spanning primers were designed. The following genes were analyzed: galectin 3 (Lgals3, F- ccattcaaaaatacaagtctctggtg, R- ccgccctctggtcttagat), vinculin (Vcl, F- agcaggccacagagatgctg, R- ccagccagatttgacgaggtg), adrenomedullin (Adm, F- ggcgctaagtcgtgggaaga, R- cttcgtctgattgctggtg), natriuretic brain peptide (Nppb, F- ggcctcacaaagaacacccaaa, R- ttctacaacaacttcagtgcgttac). As a reference, gene encoding prolyl 4-hydroxylase, beta polypeptide (P4hb, F- aagtgcagacagaacggcattg, R- gatcgtcgtcttctcatgctc) was used. Expression of this gene had been confirmed by microarray data analysis as having low variance and showing no differences between hypoxic and control samples (data not shown). Analysis was performed using nine hypoxic and six control samples (from “true hypoxia” experiments). Real-

time PCR was performed with detection of transcripts by SYBR Green, using one-step MasterAmp Kit (Epicentre). The reactions were carried out using ABI Prism 7700 (Applied Biosystems) in 20 ml volume containing 0.04 mM forward and reverse primers and 50 ng of total RNA. The cycling conditions were: 60°C for 30 min initial denaturation, followed by 40 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s. Reactions were performed in duplicate. Expression of the examined genes was normalized to that of reference gene (P4hb).

RESULTS

Preliminary Analysis of Hypoxia Data

Three methods to achieve hypoxic conditions in cell culture vessels were used (see Materials and Methods). First we confirmed the reliability of our experiments by performing hierarchical clustering of the obtained samples, based on known hypoxia-regulated genes (see Fig. A, Web Appendix, <http://www.genomika.pl/cancerbiology/hypoxia>). We further compared gene expression in hypoxic samples with that in control ones using 1) fold-change approach, 2) Welch *t*-test with False Discovery Rate estimation (FDR), and 3) a linear model with Bayesian moderated *t*-statistic (*limma*). Each method yielded large, but slightly different, sets of genes (Table 1). The lists were overlapping to a great extent, especially for three “true hypoxia” experiments and for two CoCl₂ concentrations used.

Pattern Discovery by Singular Value Decomposition

There were two main sources of variance in the performed experiments: effect of treatment (hypoxia vs. normoxia) and different means of inducing hypoxia (regulated or nominal 1% oxygen vs. chemical hypoxia mimicry). We used SVD method to delineate the experimental groups and compare the effect of hypoxia with commonly used methods of its induction. Samples clustered into distinct subclasses, which mainly represented the two mentioned sources of variance (Fig. 1). Using an SVD algorithm, we carried out unsupervised gene selection to extract probesets representing all major patterns of variability. The genes with expression profile similar to the first four SVD modes were used for hierarchical clustering of the samples (Fig. 2).

For the first three modes we obtained more than 100 probesets per mode (154, 134, and 125, respectively) whereas for the fourth mode 35 probesets were selected. The first mode genes subdivided

TABLE 1
GENES DIFFERENTIATING BETWEEN HYPOXIC AND CONTROL SAMPLES

	True Hypoxia			Hypoxia Mimicry	
	O ₂ -Regulated Incubator 1 (n = 5)	O ₂ -Regulated Incubator 2 (n = 5)	Billups-Rothenberg Chamber (n = 5)	100 μ M Cobalt Chloride (n = 4)*	200 μ M Cobalt Chloride (n = 4)*
Twofold change	169/70	168/46	123/147	236/713	311/705
Fourfold change	36/1	40/0	27/0	44/116	56/117
FDR <5% (<i>limma</i>)	781/1036	737/750	759/1427	1983/2116	2190/2286
Twofold change (mean)		145/56		196/667	
FDR <5% (Welch test)		1016/1525		105/259†	

Probesets were selected by fold-change criterion (two- or fourfold, mean fold change was calculated either in each experiment separately or for three “true hypoxia” and two hypoxia mimicry experiments) or by statistical selection (*limma*, Bayesian moderated *t*-statistic for each experiment or classic Welch *t*-test for larger groups). False Discovery Rate (FDR) was estimated by Benjamini-Hochberg method. Numbers of probesets satisfying the criteria are given as “upregulated/downregulated.”

*Two samples treated with 100 μ M CoCl₂ and two treated with 200 μ M CoCl₂ were compared to common controls (two samples).

†Relatively low number of genes due to small number of samples compared.

treated samples (1% O₂ and CoCl₂) from controls, confirming that hypoxia was the strongest factor influencing gene expression in the analyzed dataset (see Fig. 2A). The second mode classified “true hypoxia” samples together with control ones versus CoCl₂-treated samples (Fig. 2B). This indicated that there was a considerable difference between “true hypoxia” and hypoxia mimicry-induced expression pro-

files. The third mode genes showed the same classification of samples as the second mode probesets but with different pattern of gene expression (Fig. 2C). The fourth mode subdivided samples into two groups: one including O₂-regulated incubator samples together with those exposed to 200 μ M CoCl₂ and the other containing controls, Billups-Rothenberg chamber samples, and those exposed to 100 μ M

Figure 1. Singular Value Decomposition analysis of samples. The unsupervised analysis revealed distinct differences between control and treated samples (first principal component, 39.76% of variance) and showed other subdivisions between samples, mainly related to CoCl₂ versus “true hypoxia” difference. A: control samples (open circles); B: “true hypoxia” samples (black circles: O₂-regulated incubator samples; dark gray circles: Billups-Rothenberg chamber samples); C: cobalt chloride samples (light gray circles: 100 μ M CoCl₂ samples; checkered circles: 200 μ M CoCl₂ samples).

Figure 2. Hierarchical clustering of samples. The genes with expression profile similar to the first four SVD modes were used. The analysis revealed several different patterns of variability within analyzed samples. (A) First mode: control and treated samples show subdivision into two distinct branches. (B) Second mode: CoCl₂-treated samples cocluster with controls. (C) Third mode: pattern of clustering similar to that of the second mode. (D) Fourth mode: O₂-regulated incubator samples and 200 μM CoCl₂ samples cluster into distinct branches.

CoCl₂ (Fig. 2D). In summary, SVD analysis confirmed that the difference between hypoxic and control samples was the strongest source of variance in our dataset; it also indicated that gene expression profile induced by hypoxia mimicry differs from that obtained under “true” hypoxic conditions.

Difference Between True Hypoxia and Hypoxia Mimicry

The unsupervised analysis (Figs. 1 and 2) indicated distinct differences in gene expression profile between “true” hypoxia-exposed and cobalt chloride-treated cells.

We decided to compare all experimental datasets to identify genes that were shared and those that were unique for true hypoxia or hypoxia mimicry. We compared the individual gene lists obtained from treated versus control comparison for each experiment (five in total, see Table 1). The analysis was limited to 1000 highest rank probesets (all of them were significant, with FDR <5%) selected by *limma* method. There were 203 transcripts common to all five sets, 414 genes specific only for “true hypoxia” experiments (not occurring in any of the CoCl₂ lists), and 219 genes present in hypoxia mimicry comparisons while absent from “true hypoxia” experiments. The results of the analysis are shown in the Web Appendix (Table B). The first gene group comprised genes regulated by HIF-1 transcription factor (e.g., *Vegfa*, *Bnip3*, *P4ha2*, *Slc2a1*). We found some important genes to be differentially regulated in hypoxia-exposed and cobalt-treated cells. These were *Itga4*, *Anxa1*, and *Mitf*, which were upregulated by true hypoxia while downregulated by cobalt ions.

To understand the biological impact of the observed effects of true hypoxia and hypoxia mimicry we performed ontology comparison of respective gene lists: exclusive for “true hypoxia” (414 genes, see above) and exclusive for cobalt chloride (219 probesets) (see Table B, Web Appendix). True hypoxia significantly upregulated 29 gene groups associated mainly with glycolysis, migration, adhesion, MAPK activity, and others. The gene groups upregulated by cobalt chloride were much less numerous and were affiliated with immune response (antigen presentation, see Table C, Web Appendix). The analysis of downregulated genes revealed one GO class significantly suppressed by true hypoxia (dicarboxylic acid transport) and 51 gene groups affected by cobalt chloride. They were related mainly to: proliferation (e.g., cell cycle, mitosis, DNA metabolism), DNA repair, and protein modifications (see Table C, Web Appendix). To sum up, gene ontology-based differentiation between “true hypoxia” and hypoxia

mimicry revealed many crucial gene groups upregulated only by true hypoxia. Cobalt ions, on the other hand, significantly suppressed transcription of genes involved in proliferation, DNA repair, and protein modification; similar suppression was not observed to such an extent under true hypoxia.

Hypoxia-Regulated Genes in B16(F10) Murine Melanoma Cells

Due to the pattern of observed differences between cobalt and true hypoxia datasets we restricted further analysis to the latter. Stringent Family-Wise Error Rate estimation selected 454 probesets differentiating between hypoxic and normoxic samples. Within this list 216 transcripts were upregulated (three genes upregulated more than 10-fold and 99 probesets with fold change >2), while 238 were downregulated (most suppressed *Sfrs1* with 3.5-fold change and 37 probesets with fold change >2) (Table 2). Among the upregulated transcripts there were known hypoxia-responsive genes (*Vegfa*, *Adm*, *Lgals3*, *Bnip3*, *Pfkfb*) as well as genes that, to our knowledge, were not reported as hypoxia related (e.g., *Itpk1*, *Kctd12*, *Nme1*, *Selenbp1*). The full list is presented in the Web Appendix (Table A). To validate our microarray data, the expression of four selected genes that have not been reported previously in melanoma as hypoxia associated was evaluated by quantitative RT-PCR (Q-PCR). These were genes coding for: galectin-3, vinculin, natriuretic brain peptide, and adrenomedullin. The Q-PCR results were in agreement with microarray data, although the respective fold-changes were much higher (Table 3).

To bring out biological role of the selected transcripts gene ontology analysis was performed. The most overrepresented ontology class among the upregulated genes was energy derivation by oxidation of organic compounds. This class was mainly represented by glycolytic transcripts (e.g., *Pfkfb*, *Hk2*, *Aldo3*, *Eno2*), but also comprised genes regulating glycogen metabolism (*Gys3*, *Gbe1*) and gluconeogenesis (*Gpi1*). Highly upregulated was peptidyl-amino acids modifications class including known hypoxia-regulated transcripts *P4ha1* and *P4ha2* (6,11, 17). Significant upregulation was also observed for regulation of apoptosis, antigen presentation, and regulation of transcription and rhythmic processes (Table 4). Regulation of apoptosis class included both negative (*Vegfa*, *Cdkn1a*, *Bcl2l1*, *Bcl2*) and positive (*Bnip3*, *Bnip3l*, *Bnip1*, *Jun*) regulators. The immune response genes (antigen presentation) were represented by MHC class I transcripts (*H2-B1*, *H2-D1*, *H2-K1*). Highly overexpressed within the regulation of transcription class were negative regulators:

TABLE 2
TRUE HYPOXIA-REGULATED GENES IN B16(F10) MURINE MELANOMA CELL LINE

Affymetrix Probeset ID	Gene Symbol	Gene Name	Fold-Change (Hypoxia vs. Control)
Top 20 upregulated probesets			
95706_at	Lgals3	lectin, galactose binding, soluble 3	19.41
100596_at	Selenbp1*	selenium binding protein 1	13.65
104184_at	Nppb	mouse BNP gene for brain natriuretic peptide, complete cds.	13.08
98478_at	Ccng2	cyclin G2	7.985
93294_at	Ctgf	connective tissue growth factor	7.468
104337_f_at	Pkp2	plakophilin 2	6.967
97834_g_at	Pfkip	phosphofructokinase, platelet	6.943
93836_at	Bnip3	BCL2/adenovirus E1B 19 kDa-interacting protein 1, NIP3	6.936
103520_at	Vegfa	vascular endothelial growth factor A	6.138
102798_at	Adm	adrenomedullin	6.023
160495_at	Ahr	aryl-hydrocarbon receptor	5.765
94004_at	Cnn2	calponin 2	5.528
104338_r_at	Pkp2	plakophilin 2	5.442
93738_at	Slc2a1	solute carrier family 2 (facilitated glucose transporter), member 1	5.364
101551_s_at	Tes	testis derived transcript	5.151
98496_at	Gys3	glycogen synthase 3, brain	5.134
96803_at	Gbe1	glucan (1,4- α -), branching enzyme 1	5.016
104701_at	Bhlhb2	basic helix-loop-helix domain containing, class B2	4.742
100509_at	Rnf19*	ring finger protein (C3HC4 type) 19	4.64
97870_s_at	Ero1l	ERO1-like (<i>S. cerevisiae</i>)	4.416
Top 20 downregulated probesets			
104586_at	Sfrs1	splicing factor, arginine/serine-rich 1 (ASF/SF2)	0.282
103821_at	Cdc6	cell division cycle 6 homolog (<i>S. cerevisiae</i>)	0.3
95063_at	Cdca7*	cell division cycle associated 7	0.34
97445_at	Ppid*	peptidylprolyl isomerase D (cyclophilin D)	0.34
103412_at	3732412D22Rik	RIKEN cDNA 3732412D22 gene	0.34
103922_f_at	Cyb5r1	cytochrome b5 reductase 1	0.38
94340_at	1110004L07Rik	exportin, tRNA (nuclear export receptor for tRNAs)	0.39
97538_at	Gus	beta-glucuronidase	0.4
93320_at	Cpt1a	carnitine palmitoyltransferase 1a, liver	0.41
103665_at	Elovl6*	ELOVL family member 6, elongation of long chain fatty acids (yeast)	0.42
95135_at	Mid1p1	Mid1 interacting protein 1 (gastrulation specific G12-like (zebrafish))	0.42
92582_at	Slc1a5*	solute carrier family 1 (neutral amino acid transporter), member 5	0.42
93237_s_at	Tyms-ps*	thymidylate synthase	0.425
160878_at	Bop1*	block of proliferation 1	0.43
95462_at	Bzw2	basic leucine zipper and W2 domains 2	0.44
99620_at	Sfpq*	splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated)	0.44
103619_a	Cyb5b	cytochrome b5 type B	0.45
96340_at	Tmem50b	transmembrane protein 50B	0.45
100026_at	Bcat1*	branched chain aminotransferase 1, cytosolic	0.45
160723_at	1500001M20Rik	RIKEN cDNA 1500001M20 gene	0.46

Genes selected by comparing the "true hypoxia" group to control samples (FWER < 0.05, Westfall-Young algorithm). Only the genes with the highest fold-change are shown (full list available in the Web Appendix, Table A).

*Genes not previously associated with hypoxia.

Bhlhb2, Sap30, hypoxia induced in fibroblast cells (22), and Mxi1, retrievable from other hypoxia studies (4,6,43,46). We also noted the upregulation of Ahr, Atf3, Stat3, and Mitf (a cell-specific transcription regulator). GO classes associated with tissue remodeling (migration, adhesion, motility) and angiogenesis did not meet, as a whole, the significance criteria applied in this study, but among genes making up those classes there were highly upregulated

transcripts such as Lgals3, Ctgf, Adm, Cnn2, Vegfa, or Anxa2.

Genes significantly downregulated under hypoxic conditions are linked to protein and amino acid metabolism (serine family amino acid metabolism, glycine metabolism, negative regulation of translation, protein complex assembly) as well as nucleotide and DNA metabolism, including DNA replication and DNA repair classes (Table 4). Within the latter group

TABLE 3
QUANTITATIVE REAL-TIME PCR VALIDATION OF MICROARRAY RESULTS

Gene	Mean Hypoxia	Mean Control	<i>p</i> -Value	Fold-Change
Adm	54.98	0.27	$p < 0.005$	206.4
Lgals3	57.87	0.52	$p < 0.005$	112.2
Nppb	138.26	1.48	$p < 0.005$	93.2
Vcl (selected by <i>limma</i> method)	16.09	1.15	$p < 0.005$	14.0

Expression of the four genes chosen from the examined microarray datasets was normalized to that of the reference gene (P4hb). The statistical significance of results was assessed using Kolmogorov-Smirnov test.

TABLE 4
ONTOLOGY CLASSES REGULATED BY TRUE HYPOXIA

Gene Ontology ID	Name	Number of Probesets	<i>p</i> -Value
Upregulated genes			
GO:0006096	glycolysis	9	1.52E-08
GO:0015980	energy derivation by oxidation of organic compounds	13	4.65E-08
GO:0019320	hexose catabolism	9	1.01E-07
GO:0042981	regulation of apoptosis	12	0.0001
GO:0006915	apoptosis	15	0.0006
GO:0019883	antigen presentation, endogenous antigen	3	0.001
GO:0043066	negative regulation of apoptosis	6	0.001
GO:0018401	peptidyl-proline hydroxylation to 4-hydroxy-L-proline	2	0.001
GO:0018193	peptidyl-amino acid modification	5	0.003
GO:0006357	regulation of transcription from RNA polymerase II promoter	10	0.003
GO:0048511	rhythmic process	4	0.004
GO:0046777	autophosphorylation	3	0.004
GO:0016540	protein autoprocessing	3	0.004
Downregulated genes			
GO:0009069	serine family amino acid metabolism	5	1.53E-05
GO:000625	DNA metabolism	21	4.77E-05
GO:0006220	pyrimidine nucleotide metabolism	5	4.85E-05
GO:0006260	DNA replication	10	6.83E-05
GO:0044237	cellular metabolism	120	7.06E-05
GO:0006396	RNA processing	15	7.57E-05
GO:0000398	nuclear mRNA splicing, via spliceosome	10	0.0001
GO:0006461	protein complex assembly	8	0.0002
GO:0006544	glycine metabolism	3	0.0004
GO:0016070	RNA metabolism	16	0.0004
GO:0006519	amino acid and derivative metabolism	13	0.0004
GO:0009063	amino acid catabolism	5	0.0005
GO:0006397	mRNA processing	11	0.0006
GO:0006281	DNA repair	9	0.001
GO:0008295	spermidine biosynthesis	2	0.002
GO:0016478	negative regulation of translation	2	0.002
GO:0009058	biosynthesis	30	0.003
GO:0019752	carboxylic acid metabolism	15	0.003
GO:0006974	response to DNA damage stimulus	9	0.004
GO:0045454	cell redox homeostasis	2	0.004
GO:0007098	centrosome cycle	2	0.004
GO:0009071	serine family amino acid catabolism	2	0.004

Gene ontology analysis of transcripts differentiating “true hypoxia” samples from control ones (454 genes with FWER < 0.05). Only chosen ontology groups are presented from among all significantly upregulated or downregulated classes ($p < 0.005$, see Materials and Methods).

there were transcripts that had already been reported (Brca1 (1), Msh2 (41), Apex1 (52) or not (H2afx, Rfc5, Sfpq, Prp19) as hypoxia related. Our results show that the response to hypoxia in B16(F10) cell cultures involves common low oxygen-responsive functional categories such as metabolism, proliferation, apoptosis, regulation of transcription and also, though to a lesser extent, motility/migration, adhesion and angiogenesis. They also reveal involvement of processes that are not typically associated with hypoxia, such as antigen presentation or biosynthesis of biogenic amines (e.g., spermidine).

DISCUSSION

Comparison of Hypoxia With Cobalt Chloride

The aim of our study was to analyze gene expression profile of B16(F10) melanoma cells that had been exposed to "true" hypoxic conditions (1% O₂) or hypoxia mimicry (CoCl₂). The unsupervised data analysis has clearly demonstrated differences between expression profiles induced by true hypoxia (1% oxygen) and hypoxia mimicry (cobalt chloride). This effect was previously described by Vengellur et al. (43) for hepatocarcinoma (HepG2) cells. The authors suggested that similarity between hypoxia and hypoxia mimicry effects pertained mainly to the altered expression of glycolytic, apoptotic, and hydroxylase genes, which we also observed in our study. Among transcripts in question there are known HIF-1-induced genes (see Table B, Web Appendix), which supports the previously reached conclusion that mimicry of hypoxia response by cobalt ions is HIF-1 dependent (34). The differences observed by us suggest that, compared to true hypoxia, cobalt ions exert more antiproliferative and promutagenic effects as well as cause stronger suppression of glycoprotein metabolism and melanin biosynthesis. True hypoxia, on the contrary, seems to have a more pronounced effect on glycolysis, migration, adhesion, and MAPK activity. Thus, it seems that use of cobalt as hypoxia mimicker should be confined only to experiments investigating HIF-1 signaling pathways. Caution is suggested when interpreting cobalt-induced hypoxia results in the context of tumor environment or results obtained in whole genome profiling studies.

Biological Significance of Selected Genes

We selected 454 probesets differentiating between hypoxia- and normoxia-exposed B16(F10) cells. Among them there are known hypoxia-regulated genes such as Vegfa, Glut1, Bnip3, Adm, Lgals3, P4ha1, Mxi1, Hk2, Ndr1, and others. Some of our selected genes

have already been reported to be hypoxia regulated in melanoma cells. These are Cyr61 (selected by *limma* method) and VEGF. The first one was described as angiogenic factor constitutively expressed in highly metastatic melanoma cell lines (23). VEGF expression was upregulated in WM35 cells under hypoxic conditions (37). Its expression correlates also with melanoma growth and survival in vitro (12) as well as high tumor thickness (31).

Low oxygen tension enhances metastatic potential of B16(F10) murine melanoma cells in vivo (30). It also promotes lymph node metastases in human melanoma xenografts (33). We selected genes previously reported as related to melanoma progression, and because hypoxia was proved to induce melanoma aggressiveness this may suggest their association with hypoxia in vivo. The genes in question are galectin 3 (Lgals3), transcription factors Mitf and Stat3, melanoma cell adhesion molecule (Mcam/Muc18), and Nme1. Lgals3 was the most overexpressed gene in our study with 19-fold change (expression increase by Q-PCR was more than 100-fold). This would suggest that this protein is a strong survival factor in melanoma cells. Expression of galectin-3 in melanoma was shown to be higher in thin melanoma lesions than in benign nevi (44), and its elevated serum level was observed in patients with advanced disease (45). Galectin-3 regulates migration and adhesion; it also has antiapoptotic and proangiogenic activities. Due to its multiple functions, Lgals3 seems to be a very good therapeutic target in malignant melanoma. Mcam is known to promote melanoma progression. Its expression was shown to correlate with metastatic potential of mouse melanoma cell lines (51) and melanoma patient survival (29). No reports of its regulation by hypoxia have been published so far. Mitf gene was proposed as a survival oncogene amplified in malignant melanoma (7) and its downregulation was associated with sensitivity of melanoma cells to INF- γ therapy (10). The role of Mitf in melanoma progression is further indicated by a finding that the expression of gene encoding a Mitf expression inhibitor (EMX2) correlated with longer metastasis-free survival (48). Activity of Stat3, in turn, contributed to breast cancer cells' resistance to apoptosis and correlated with patients' resistance to chemotherapy (13). This appears interesting in the context of hypoxia-induced resistance to various therapeutic modalities. Activated Stat3 was also proved to promote melanoma brain metastasis (50). Contribution of hypoxia to prometastatic phenotype of melanoma seems to be supported also by our data concerning downregulation of the known metastatic suppressor gene Nme1. High level of the protein encoded by this gene was associated with decreased metastatic potential of

melanoma cells (14). To our knowledge, this is the first report of its association with hypoxia. Due to their biological function and previously established link to cancer, the described genes seem to merit further validation. However, the results of Ord et al. indicate that in vivo validation of the hypoxia results obtained in vitro is more likely for highly changed genes than for genes with lower fold change (28). The most upregulated genes in our study were galectin 3, selenium binding protein 1 (Selenbp1), and brain natriuretic peptide (Nppb). Selenbp1 was not correlated with hypoxic stimulus before, whereas in our dataset its expression under hypoxic conditions is 13 times higher than under normoxia. This may indicate its possible role in attenuating adverse effects of reactive oxygen species (by binding selenium, a known antioxidant) produced under hypoxic conditions. Reduced expression of selenium binding protein 1 was found correlated with poor outcome in patients with lung adenocarcinoma (3) and ovarian cancer (18). Highly expressed in our dataset also were adrenomedullin (Adm), a potent angiogenic factor expressed in many cancers (5), as well as Ctgf, frequently associated with various cancer types [see,

e.g. (25,49)]. These genes, in our opinion, seem to particularly merit further validation.

Our results may also indicate a possible hypoxia-responsive enhancement mechanism in B16(F10) involving Ilk, Mitf, and Stat3 genes. Mitf was reported to stimulate HIF-1 α gene expression in B16(F10) melanoma cells (2), and Ilk was shown to stimulate HIF-1 α expression in prostate cancer cells (40). In our study these genes were upregulated (1.9-fold change for Ilk, 2-fold for Mitf, and 1.6-fold for Stat3). This could possibly indicate the existence of a positive feedback mechanism enhancing hypoxia response, especially if these genes are also HIF-1 regulated. Stat3 has already been proved to be activated under hypoxic conditions and, at the same time, stabilize HIF-1 α in renal carcinoma cells (21).

Molecular Signature of Hypoxia

Previous studies have shown significant heterogeneity in hypoxia response among various cell lines (4,6). Here, we present analysis of hypoxia impact on transcription in B16(F10) murine melanoma cells and compare our results with those obtained using similar

TABLE 5
EXPRESSION OF CHOSEN HYPOXIA-REGULATED GENES IN VARIOUS HYPOXIA STUDIES

Gene	Present Study (Fold-Change)	Greijer et al. (11) (Fold-Change)	Vengellur et al. (43) (Fold-Change)	Denko et al. (6) (Present/ Absent)	Chi et al. (4) (Present/ Absent)
Vegfa	6	1.2	5	–	–
Glut1	5.4	NS	NS	+	+
P4ha1	2.4	NS	3.2	–	+
P4ha2	3.4	NS	NS	–	+
Bnip3	6.9	4.8	4.4	BNIP3L*	BNIP3L*
Ndrp1	3.1	3.2	78	+	+
Wsb1	2	–2.4	3.9	–	–
Aldo1	NS	3.2	2.5	+	–
Aldo3	3.7	3.1	NS	+	–
p21	4	NS	NS	+	–
Mxi1	3.6	NS	NS	+	+
Pfkfb	5	4.5	NS	PFK†	+
Adm	6	NS	6.8	–	+
Epo	NS	NS	NS	–	–
NO2	NS	NS	NS	–	–
Ccng2	8	NS	NS	+	–
Gbe1	5	NS	NS	–	–
Gys3	5.1	2.3	NS	–	–
Ldh1	NS	3.4	NS	+	+
Eno1	Eno2†	2.3	NS	Eno3†	–
HIG1	3.3	3.2	NS	–	–
PAI1	NS	NS	14.2	–	–

NS: not significant.

*Functional homolog of Bnip3.

†Cell type-specific isoenzyme.

experimental approaches, but different cell lines (4,6,11,43). For comparison purposes we analyzed, using our experimental data, the hypoxia signatures derived by Greijer et al. [(11), Tables 1 and 2] and Vengellur et al. [(43), Supplemental Table]. The former authors specified 103 hypoxia-responsive genes in murine fibroblasts. Thirty-eight genes from that signature were also differentially expressed in our experiments (13 out of them are present in our list of 454 selected transcripts; see Table A, Web Appendix). They are responsible for known hypoxia-responsive processes such as glycolysis (e.g., Aldo3, Pfkfb, Gpi1, and Hk2), oxydoreductive processes (Grhpr, P4ha2, and Egl1), apoptosis (including most overexpressed genes in our study: Lgals3 and Bnip3), protein transport (Kdelr3), and signal transduction (Wsb1, Rock2). The similarity between Greijer's results and ours extends also to the expression of prolly hydroxylases and genes associated with cytoskeleton organization, although a differing set of the latter class of transcripts was obtained. In turn, 57 genes from hypoxia signature obtained by Vengellur et al. (43) for Hep3B cell line were also differentially expressed in our experiments (11 genes common with our initial list of 454 selected transcripts). The genes in question are involved in glycolysis (Ldh1, Aldo1), cell cycle regulation (Tfdp1, Rfc3), apoptosis (Bnip3, Bnip3l, Jun), protein biosynthesis (Mrps11, Mrps12), protein metabolism (P4ha1, Uchl1), and protein folding (Dnaja1, Bag2, Abce1) (see Table D, Web Appendix). We also compared directly our results with those of Denko et al. (7) and Chi et al. (4). The results are shown in Table 5.

It appears that, despite common cellular functions modulated by hypoxia as well as some overlapping of transcripts, differences in hypoxia response between cell lines may be more significant than similarities. Moreover, expression of hypoxia markers may vary depending on pH and actual oxygen concentration (39). Also, methodology of studies as well as specific cell lines used (even of similar anatomical origin) affect final results (42). This may help explain why no reliable hypoxia markers have been found so far. It seems that only sets of genes (signatures) regulated by hypoxia in a particular cancer type are likely to be relatively reliable indicators of hypoxic state. Chi et al. (4) have shown that a set of 168 genes induced by hypoxia in several epithelial cell lines properly classifies clinical material according to high or low hypoxia response; this signature has also correlated with patients' survival. It seems warranted, therefore, to investigate hypoxia response in at least tissue-specific context and validate the expression of selected genes in clinical material.

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