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Microarray-based Analyses of Hypoxia-induced Transcriptional Changes in Breast Cancer Cell Lines

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Abstract. *Background: Tumour hypoxia is a common characteristic of many solid human tumours, and is associated with a poor prognosis in various types of cancer. Metabolic changes occur when cells are exposed to low oxygen pressure; however, little is known about the mechanisms underlying malignant transformation and/or progression caused by hypoxia. Materials and Methods: We monitored global gene expression changes caused by hypoxia in four breast cancer cell lines using 27K cDNA microarrays. Cells were grown under hypoxic and normoxic conditions, and were harvested at four different time points. All genes were assigned to patterns (up, down, or unchanged) across the time points, followed by ontological mapping to investigate significant associations between genes belonging to specific patterns and Gene Ontology categories. Furthermore, we investigated genomic regions upstream of regulated genes for the presence of known regulatory motifs. Results: Several common effects of hypoxia were seen in the breast cancer cell lines, such as an increase in glycolytic metabolism; however, the response to hypoxia varied greatly between the cell lines. Oestrogen receptor (ER)-positive breast cancer cells displayed a partially unique response to hypoxia compared to ER-negative cells. Similarly, unique changes in e.g. RNA metabolism and DNA repair were seen in a BRCA1-deficient cell line. Whereas an enrichment of genes containing the HIF-1 binding site sequence was found among genes regulated by hypoxia in two of the cell lines investigated, this sequence was also identified in a considerable fraction of non-regulated genes. Conclusion: Global gene expression profiling of the cellular response to hypoxia revealed a multitude of novel mechanisms and functions affected by hypoxia in breast cancer cell lines. The findings also suggest a high degree of diversity in this response depending on the genetic background of the tumour cells. Specifically, down-regulation of genes involved in DNA repair mechanisms in a BRCA1-deficient cell line may reflect the crucial role played by the BRCA1 protein in instances of DNA damage, e.g. during hypoxia.*

Areas with low oxygen pressure, or hypoxia, are a common feature in most solid human tumours (1). Hypoxia develops when the blood supply is limited due to poorly organised or occluded blood vessels, or insufficient compared with the growth of malignant cells. Therefore, in order to grow beyond the size of a few millimetres, solid tumours require neovascularisation (2, 3). Nearly 50% of locally advanced breast cancers exhibit regions of hypoxia and/or anoxia (4). Hypoxia is associated with a poor prognosis in many types of cancer, partly due to an increased genetic instability and mutation frequency (5), and increased metastatic potential (6), and partly due to reduced sensitivity to both ionising radiation and chemotherapy (7). The transcription factor hypoxia-inducible factor-1 (HIF-1), composed of the O_2-regulated HIF-1α and constitutively expressed HIF-1β subunits (8), is the key regulator of oxygen homeostasis (9), mediating the activation of *e.g.* angiogenic factors (2). HIF-1 target genes encode proteins that increase O_2 delivery and mediate adaptive responses to O_2 deprivation (2). Over 40 HIF-1 target genes have been identified thus far, which encode proteins that play key roles in important biological processes including angiogenesis, glucose transport, glycolysis, cell proliferation, and apoptosis (reviewed in (10)). Although HIF-1 expression is regulated primarily on the protein level, many HIF-1 target genes are

Abbreviations: BM, bone marrow micrometastasis; BRCA1, breast cancer gene 1; ER, oestrogen receptor; HIF-1, hypoxia-inducible factor 1; HRE, hypoxia responsive element.

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believed to be transcriptionally regulated. In order to investigate the time-dependent transcriptional regulation of hypoxia-responsive genes, we performed time-course experiments in four different breast cancer cell lines subjected to hypoxia and compared these with cells grown under normoxic conditions using 27K cDNA microarrays.

Ontological mapping of the microarray findings revealed a large number of genes involved in a variety of biological processes that were regulated in a time-dependent manner by hypoxia. Of interest, only a limited fraction of the regulated genes were in common between the cell lines, the majority of which have previously been shown to be regulated by hypoxia (10). In addition to this general response, each cell line displayed a unique response to hypoxia, part of which may be explained by differences in oestrogen receptor (ER) expression or function of the breast cancer susceptibility gene *BRCA1*. We investigated whether the regulatory motif associated with hypoxia, the hypoxia responsive element (HRE), was associated with genes regulated by hypoxia by analysing the presence of the HIF-1 binding site sequence, and found an overabundance of genes containing this motif in hypoxia-regulated genes in two of four breast cancer cell lines, *i.e.* there was an association between genes containing the specific HIF-1 binding site sequence and hypoxic conditions. Of note, however, the magnitude of this overabundance was only moderate, and all cell lines exhibited a considerable number of non-hypoxia-regulated genes with this motif.

Materials and Methods

Cell lines. The breast cancer cell lines MDA-MB-468, MCF-7 and T47D were obtained from ATCC (Rockville, MD, USA). The cell line L56Br, derived from a lymph node metastasis from a woman with a truncating *BRCA1* mutation (1806C>T) was recently established (11). Cells were grown in RPMI 1640 (MDA-MB-468, MCF-7 and L56Br) or D-MEM high glucose (T47D) medium supplemented with 10% FCS, 1mM Na-pyruvate (MDA-MB-468 and L56Br), 1.5g/l Na-bicarbonate (MDA-MB-468), 10 mM Hepes (T47D and L56Br), non-essential amino acids (L56Br), 10 μg/ml insulin (MCF-7, T47D and L56Br) and 50 IU/ml penicillin and 50 μg/ml streptomycin. Cell cultures were sub-cultured weekly, and medium was exchanged twice in-between. Cell cultures were grown at 37˚C in a humidified atmosphere containing 5% CO_2 in the air.

Experimental design. Cells were seeded at low density one day prior to the start of treatment and the medium was exchanged at time point zero. Hypoxia-treated cells received medium subjected to 1% O_2 and were further cultured in a humidified chamber containing 1% O_2 and 5% CO_2 at 37˚C. Control cells were cultured in an atmosphere of 21% O_2 and 5% CO_2 at 37˚C. Hypoxia-treated and control cells were harvested at 6, 12, 24, and 48 hours after time point zero. Cells were rinsed once with PBS and stored in TRIzol (Life Technologies Inc., Rockville, MD, USA) at -80˚C in preparation for RNA extraction.

Microarray procedure. Total RNA was extracted using TRIzol followed by RNeasy (Qiagen, Valencia, CA, USA), according to the manufacturers' recommendations. RNA was reverse transcribed and labelled by indirect labelling using the CyScribe Post-Labelling Kit (Amersham Biosciences Corp, Piscataway, NJ, USA), according to the manufacturer's recommendations. Labelled cDNAs were purified using GFX columns (Amersham Biosciences), dried and resuspended with blockers in hybridisation buffer from Pronto! Plus Systems (Corning Inc., Corning, NY, USA). Custom-made cDNA microarrays containing approximately 27,000 clones in duplicate (representing 17,517 unique UniGene clusters) were obtained from the SWEGENE DNA Microarray Resource Center at the BioMedical Center B10 in Lund, Sweden, supported by the Knut and Alice Wallenberg foundation through the SWEGENE Consortium. Slides were hybridised at 42˚C, washed and scanned using an Agilent DNA Microarray Scanner (Agilent Technologies, Palo Alto, CA, USA), and image analysis and data extraction were performed using the GenePix Pro 4.0 software (Axon Instuments Inc., Union City, CA, USA). One hybridisation was performed for each cell line and time point, and RNA from time point zero from each cell line was used as a reference for that cell line.

Data analysis. Pre-processing. The data extracted using GenePix was stored in BASE (12), and BASE was used for quality control and normalisation. Briefly, based on the parameters extracted by the GenePix software, our quality filter required each spot to have a signal-to-noise ratio of at least 3 for both channels (Cy3 and Cy5), a diameter of at least 7 pixels, and flag equal to zero. Spots that did not fulfil all of these requirements were treated as missing values in the subsequent analyses. Each experiment was subsequently normalised using LOWESS regression as implemented in BASE. To get a relative expression level caused by hypoxia for each gene, the intensity ratio in the hypoxia-treated sample was divided with the intensity ratio in the untreated sample. These relative expression levels were converted into four discrete levels (+, up-regulated as compared to the untreated sample; -, down-regulated; 0, unchanged; m, missing value) based on cut-offs from self-self hybridisations. The cut-offs were individually selected for each cell line such that 5% of the genes in a comparison of two self-self hybridisations (RNA extracted and harvested from the same source at different times) were expected to be either + or -.

Pattern analysis. Using this four-valued representation, each gene was assigned a pattern across the four time points, for example, ++++ for a gene that was up-regulated at 6, 12, 24 and 48 hours. We assigned a gene to the up-early category if it belonged to the ++++ pattern or any other pattern with one of the time points changed from + to 0 or m. Genes were assigned to the up-late category if they belonged to the 00++ pattern or any other pattern with one of the first two time points changed from 0 to – or m. Similarly, we defined down-early genes based on the ----, and down-late genes based on the 00-- patterns, respectively. Because we required at least three time points with a + for a gene to belong to the up-early category, we would expect approximately $(2.5\%)^3=0.002\%$ of the genes on the arrays to belong to this pattern if all four time points had been ratios between self-self hybridisations. For the up-late pattern, we would expect $(2.5\%)^2=0.06\%$ and, correspondingly, for the down-early and down-late patterns. Taken together, these percentages imply that our cut-offs resulted in conservative assignments of genes to patterns across time-points, *i.e.* low numbers of false-positives were expected.

Gene ontology analysis. We used the publicly available GoMiner software (13) to investigate significant associations between genes belonging to specific patterns and Gene Ontology categories (14).

HRE motif analysis. We investigated genomic regions upstream of transcripts for the presence of known regulatory motifs. For each gene, we extracted a transcript in the UniGene cluster (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene) associated with the gene (using build 160 of UniGene). If there was a RefSeq mRNA sequence in the cluster it was used; if not, the longest other mRNA sequence was used. The positions of the transcripts in the July 2003 human reference sequence (UCSC version hg16) of the Santa Cruz Genome Database (15) were used to define transcription start sites. In the analysis, we searched for the presence of the consensus motif 5'-G/C/T-ACGTGC-G/T-3' (16) 10,000 basepairs upstream and 1,000 basepairs downstream of transcription start sites. We used such a large region because the transcription start sites are only approximate. We identified 8,550, 4,612, 5,806, and 4,203 genes after quality filtering with upstream regions in the four cell lines MDA-MB-468, MCF-7, T47D, and L56Br, respectively. To investigate if associations between hypoxia-responsive genes and the presence of the HIF-1 binding site sequence were significant, Fisher's exact test was used on 2x2 contingency tables.

Results

Time-dependent transcriptional changes caused by hypoxia. Cells were seeded such that they were in exponential growth at the onset of hypoxia treatment, and did not reach confluency before the end of the experiment. No significant differences in S-phase fraction or apoptotic cell death between normoxia and hypoxia were seen for the duration of the experiment in any of the cell lines (data not shown). Of note, though, there were differences in growth rate between the cell lines, such that MDA-MB-468 was the fastest growing and MCF-7 and T47D more slow growing. Hypoxia caused major transcriptional changes in the three breast cancer cell lines MDA-MB-468, MCF-7 and T47D. Hypoxia-responsive genes were divided into four categories; genes up-regulated early (at 6 and 12 hours), genes up-regulated late (at 24 and 48 hours), and, correspondingly, genes down-regulated early or late after onset of hypoxia. Specifically, only a small number of genes, illustrated by the overlap in the middle of each panel, were found to be similarly regulated in all cell lines (Figure 1A-D). Many of these genes have previously been shown to be regulated by hypoxia (17), and include glycolytic enzymes and transporters (enolases 1, 2, and 3, synaptoporin and aldolases A and C), and genes involved in maintenance of cytoskeletal structure (keratin14) (Figure 2). Other genes induced by hypoxia in our study include the developmental genes *SEMA4B* and *SCN8A*, the apoptosis-inducing gene *FOXO3A*, the DNA-binding gene *MLL5*, and metal ion-binding genes *S100P* and *ERO1L* (Figure 2). In addition, genes involved in transcriptional regulation (*NOL5A* and *NCOA5*), nucleic acid metabolism (*ZNF207, DKC1* and *POLE3*), differentiation (*NOTCH2*), and cell cycle regulation and proliferation/apoptosis (*ILF3*) were found to be down-regulated by hypoxia in all three cell lines while the cell death promoting gene *PORIMIN* was up-regulated (Figure 2). The overlap of genes regulated by hypoxia increased when the time dependency was excluded (Figure 1E-F), suggesting that the time dependence of the response to hypoxia may depend on the cell type, possibly due to differences in proliferation rate in the different cell lines. In addition to the overlapping transcriptional changes, representing a general, highly conserved, response to decreased oxygen tension, each cell line showed an extensive and unique response to hypoxia (Figure 1A-D).

Ontological mapping of hypoxia-induced transcriptional changes. In order to investigate if any particular cellular or molecular processes or functions were affected by the hypoxic conditions in the breast cancer cell lines, we used the Gene Ontology software GoMiner (13) to map genes to ontological pathways. Genes were divided into those up-regulated by hypoxia (Table IA), and those down-regulated by hypoxia (Table IB). Among the processes involving up-regulated genes, general metabolic effects of hypoxia treatment were seen in all cell lines investigated, including alterations in glucose and alcohol metabolism (Table IA). Genes involved in developmental and structural processes, *e.g.* histogenesis, cell differentiation and the cytoskeleton, were also found to be up-regulated by hypoxia (Table IA). An acute phase immune response could be detected in one of the cell lines (MDA-MB-468), and oxidoreductase-related genes were up-regulated in all cell lines, illustrating the adaptive response to oxygen deprivation (Table IA). Genes involved in metal ion binding (*e.g. JAG2* and *NOTCH2* and *ERO1L*) were induced by hypoxia in all three cell lines investigated, possibly indicating an increase in metastatic potential (Table IA). In this context, an enrichment of cell adhesion genes (*e.g. CD44*) was seen in two cell lines (MCF-7 and T47D). Further, an enrichment of genes encoding catalytic enzymes (*e.g.* aldolases and enolases) was seen in all three cell lines (Table IA). Hypoxia caused down-regulation of genes involved in the proteasome complex (*e.g.* 26S proteasome subunit genes) specifically in the two ER-positive cell lines MCF-7 and T47D (Table IB). In addition, metabolic effects, such as down-regulation of mRNA splicing genes (*e.g.* splicing factor *SFRS* genes and small nuclear ribonucleoprotein *SNRP* genes) and rRNA processing genes (*e.g. DKC1, NOL5A* and *DDX21*) were also seen in all cell lines (Table IB). Finally, cell cycle-related effects, particularly down-regulation of genes involved in cell cycle regulation, including down-regulation of a variety of cyclins and kinases (*e.g. CCND1, CCNE1, CDK4* and *CDC25B*), were seen mainly in MDA-MB-468 cells (Table IB). Overall, general effects on metabolic

functions, such as glucose metabolism and histogenesis, as well as oxygen metabolism, were seen in all three cell lines after exposure to hypoxia. In addition, each cell line displayed individual unique responses to hypoxia, suggesting a high degree of diversity in the response of tumour cells to the hypoxic environment (discussed below).

Unique response in oestrogen receptor-positive cell lines. It has previously been suggested that low levels of oxygen may induce a state of dedifferentiation in *e.g.* neuroblastoma (18). In addition, the occurrence of a dedifferentiated phenotype has been reported in ductal carcinoma *in situ* of the breast, involving up-regulation of keratin 19, among other proteins (19). In this context, a specific and significant induction of genes involved in developmental processes such as histogenesis, morphogenesis and organogenesis (*e.g. KRT14*) was seen primarily in the two ER-positive cell lines T47D and MCF-7 (Table IA). Overall, a total of 226 genes were found to be regulated (55 up- and 171 down-, respectively) in the two ER-positive cell lines, but not in the ER-negative MDA-MB-468 cells. However, many of these genes display a slight variation in the same direction in MDA-MB-468 cells as in the ER-positive cells. Conversely, 182 genes (95 up- and 87 down-, respectively) were found to be uniquely regulated in the ER-negative MDA-MB-468 cells, but not in the two ER-positive cell lines (data not shown). A common event in the regulation of hypoxia-sensitive transcriptional regulators is the targeted degradation of proteins through the ubiquitin/proteasome pathway. Interestingly, down-regulation of genes involved in the proteasomal complex was found specifically in the two ER-positive cell lines MCF-7 and T47D (Table IB). Moreover, up-regulation of genes involved in transcriptional regulation (*e.g. JUNB* and *JUND*) was more pronounced in MCF-7 and T47D cells (Table IA). Induction of genes implicated in apoptotic regulation, as well as helicase activity and RNA binding, was also caused by hypoxia mainly in the two ER-positive cell lines.

In addition to the induction of genes previously described as hypoxia-regulated, such as those involved in glucose metabolism, the ER-negative cell line MDA-MB-468 displayed down-regulation of several genes implicated in cell cycle progression as well as polyamine metabolism (Table IB), indicating a more general response or adaptation to the hypoxic environment in this cell line compared to the two ER-positive cell lines.

BRCA1-dependent response to hypoxia. In order to investigate whether the *BRCA1* gene is involved in the cellular response to low oxygen levels, we also subjected a *BRCA1*-deficient breast cancer cell line, L56Br, to a time series of hypoxia treatments. The left panel of Figure 4A illustrates the expression of all hypoxia responsive genes in L56Br cells, and the right panel illustrates the ontological mapping of those genes. In Figure 4B only genes unique to L56Br cells in the response to hypoxia are included. Interestingly, in addition to general metabolic effects seen also in the other breast cancer cell lines (Figure 4A), alterations in the expression of a variety of DNA repair genes, genes involved in RNA metabolism, and lyase activity were seen specifically in the *BRCA1*-deficient cell line (Figure 4B). The majority of these genes were down-regulated upon O_2 deprivation in the absence of functional *BRCA1*. In addition, among the up-regulated genes in L56Br cells were a large fraction of heavy metal binding genes, including several metallothioneins (Figure 4B).

Hypoxia-responsive elements in regulated genes. It is well known that many hypoxia-responsive genes contain the HRE regulatory motif. HIF-1 is known to bind to evolutionarily conserved HREs situated in the promoters of regulated genes, with the consensus motif 5'-G/C/T-ACGTGC-G/T-3' (16). Using this consensus motif, we performed a search for the HIF-1 binding site sequence in the upstream regions of all genes in the four breast cancer cell lines included in the present study. A search 10,000 basepairs upstream and 1,000 basepairs downstream of the start sites of each gene revealed a significant overabundance of genes containing the motif among genes regulated under hypoxic conditions in the MCF-7 and L56Br cell lines. Of note, however, a considerable fraction of genes not responsive to hypoxia also displayed this motif in all of the cell lines investigated (Table II). Furthermore, when a less stringent motif search was performed (with the commonly used 5'-ACGTG-3'), there was no significant over-abundance of the consensus motif among hypoxia-regulated genes in any of the breast cancer cell lines, as the motif was seen in a large portion of non-regulated genes (data not shown).

Discussion

It has recently been shown that HIF-1α is an independent prognostic factor for an unfavourable prognosis in patients with both lymph node-positive (20) and -negative (21) breast cancer. Moreover, decreased levels of ERα, combined with reduced function of the receptor, has been found in breast cancer cell lines after hypoxic treatment (22, 23), and the hypoxia-induced degradation of ERα has recently been shown to be proteasome-dependent (24). Taken together, these findings suggest that tumours may adapt to decreased levels of O_2 in different ways depending on the genetic background and phenotypic characteristics of the tumour cells.

The most striking finding in the present study was the low degree of overlap in regulated genes in the three breast cancer cell lines MDA-MB-468, MCF-7 and T47D in response to

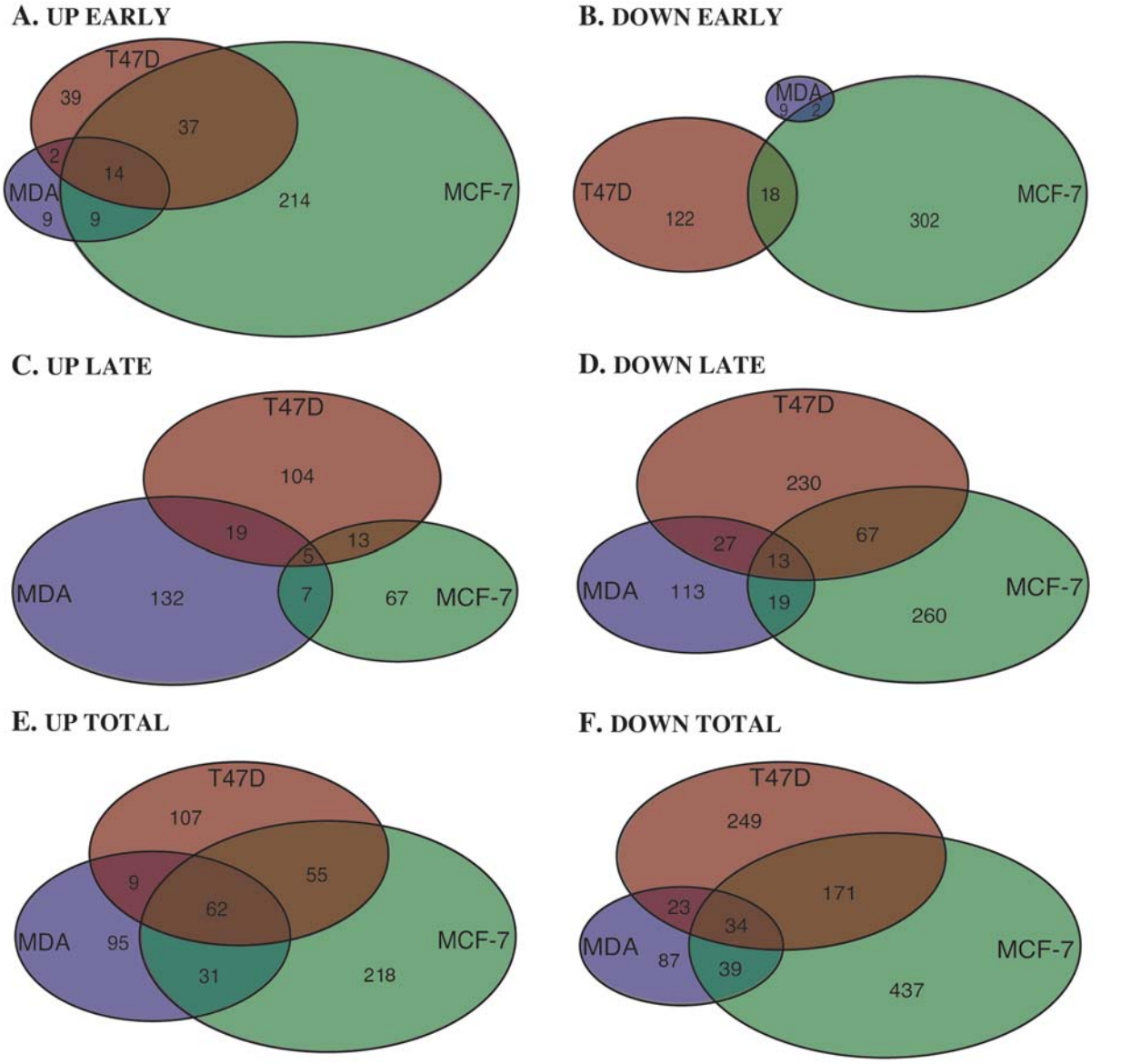

Figure 1. *Venn diagrams showing the overlap of genes regulated by hypoxia in the three breast cancer cell lines MDA-MB-468, MCF-7 and T47D. Numbers within individual circles indicate the number of clones uniquely regulated for each cell line and numbers in overlapping areas indicate genes in common between the cell lines in question. MDA=MDA-MB-468. A. Genes up-regulated at early time points (6 & 12 hours) after hypoxia. B. Genes down-regulated at early time points (6 & 12 hours) after hypoxia. C. Genes up-regulated at late time points (24 & 48 hours) after hypoxia. D. Genes down-regulated at late time points (24 & 48 hours) after hypoxia. E. All genes (time points 6, 12, 24 and 48 hours) up-regulated after hypoxia. F. All genes (time points 6, 12, 24 and 48 hours) down-regulated after hypoxia.*

hypoxia (Figure 1). However, when the early and late transcriptional events were combined, a larger fraction of genes responded similarly (*i.e.* in the same direction) in the three cell lines, suggesting that the low degree of time-dependent overlap can be at least in part explained by differences in how rapidly the cell lines respond to the hypoxic environment, and this in turn may be due to differences in replication time between the cell lines. Investigation of the molecular and functional pathways affected by hypoxia revealed considerable overlap in processes known to be affected by low levels of oxygen, *e.g.* glycolysis (Table IA). Interestingly, ontological mapping revealed a greater overlap in molecular and functional processes (Table I) than the investigation of individual genes (Figure 1), suggesting that hypoxia may affect similar functions and structures although there is a variation in the actual genes affected.

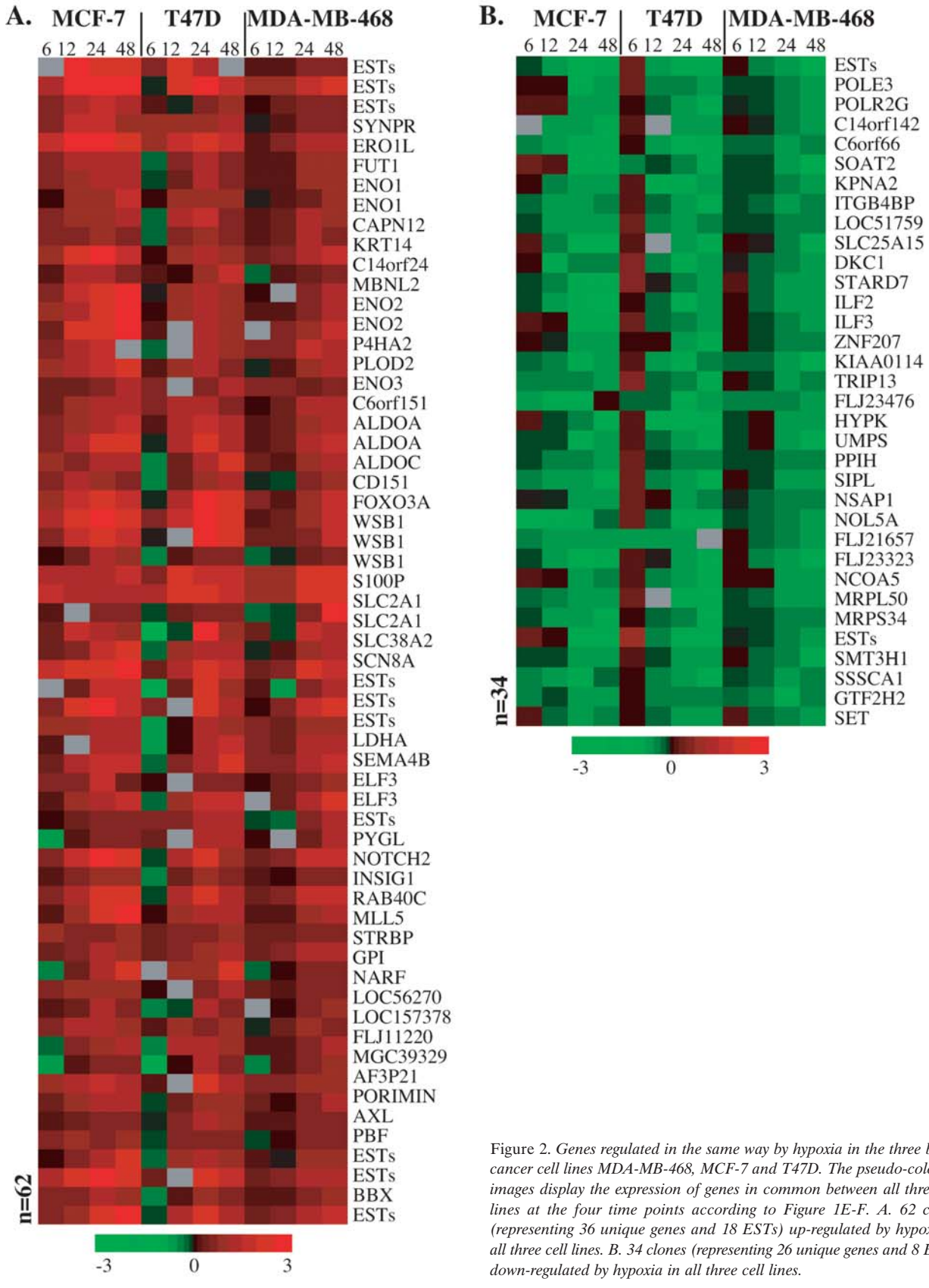

Figure 2. *Genes regulated in the same way by hypoxia in the three breast cancer cell lines MDA-MB-468, MCF-7 and T47D. The pseudo-coloured images display the expression of genes in common between all three cell lines at the four time points according to Figure 1E-F. A. 62 clones (representing 36 unique genes and 18 ESTs) up-regulated by hypoxia in all three cell lines. B. 34 clones (representing 26 unique genes and 8 ESTs) down-regulated by hypoxia in all three cell lines.*

Table IA. *Ontological mapping of genes up-regulated by hypoxia.*

Gene Ontology	MDA-MB-468			MCF-7			T47D		
	# genes	abundance	*p*-value	# genes	abundance	*p*-value	# genes	abundance	*p*-value
Biological process									
Glycolysis	**14/26**	**10.25**	**<0.001**	**12/14**	**10.45**	**<0.0001**	**9/23**	**6.52**	**<0.0001**
Histogenesis	**5/40**	**2.38**	**0.0562**	**6/13**	**5.63**	**0.0003**	**5/20**	**4.16**	**0.0055**
Cell differentiation	3/45	1.27	0.5108	**3/23**	**1.59**	**0.4291**	**5/25**	**3.33**	**0.0146**
Sterol biosynthesis	**2/17**	**2.24**	**0.2237**	0/13	na	0.6171	**5/15**	**5.55**	**0.0013**
Angiogenesis	**1/12**	**1.59**	**0.4771**	**1/3**	**4.06**	**0.2266**	**1/2**	**8.33**	**0.1165**
Regulation of transcription	29/541	1.02	0.9175	28/288	1.19	0.3020	35/376	1.55	0.0069
Immune response	12/238	0.96	1.0	**15/90**	**2.03**	**0.009**	7/132	0.88	0.8526
Acute phase response	**4/9**	**8.46**	**0.0008**	0/5	na	1.0	0/6	na	1.0
Protein modification	**34/392**	**1.65**	**0.0027**	15/200	0.91	0.7881	15/259	0.96	1.0
Iron ion homeostasis	**3/7**	**8.16**	**0.0043**	0/3	na	1.0	**1/3**	**5.55**	**0.1696**
Cell adhesion	12/195	1.17	0.5116	**13/77**	**2.06**	**0.0096**	**10/105**	**1.59**	**0.1381**
Cellular component									
Intermediate filament cytoskeleton	**6/26**	**4.39**	**0.0019**	**5/10**	**6.10**	**0.0006**	**3/13**	**3.84**	**0.0389**
Actin cytoskeleton	**7/82**	**1.63**	**0.2020**	5/47	1.30	0.5857	3/61	0.82	1.0
Microtubule cytoskeleton	**17/249**	**1.30**	**0.2409**	13/147	1.08	0.7555	10/188	0.89	0.8734
Mitochondrion	*8/274*	*0.56*	*0.0904*	*4/181*	*0.27*	*0.0010*	*4/229*	*0.29*	*0.0021*
Chromatin	0/7	na	1.0	**3/5**	**7.31**	**0.0048**	**3/6**	**8.33**	**0.0037**
Molecular function									
Lyase activity									
Carbon-carbon lyase activity	**4/19**	**4.01**	**0.0154**	**3/14**	**2.61**	**0.1010**	**3/13**	**3.84**	**0.0389**
Phosphopyruvate hydratase	**3/3**	**19.04**	**0.0001**	**3/3**	**12.19**	**0.0005**	**3/3**	**16.66**	**0.0002**
Kinase activity	**27/304**	**1.69**	**0.0068**	14/147	1.16	0.5335	16/197	1.35	0.2113
Transcription factor activity	14/288	0.93	0.8910	17/149	1.39	0.1619	**20/187**	**1.78**	**0.0098**
Metal ion binding	**40/389**	**1.96**	**<0.0001**	**27/200**	**1.65**	**0.0067**	**28/267**	**1.75**	**0.0026**
Oxidoreductase activity, paired donors	**7/24**	**5.55**	**0.0002**	**4/10**	**4.88**	**0.0062**	**6/15**	**6.66**	**0.0001**
Oxidoreductase activity, single donors	**5/9**	**10.58**	**<0.0001**	**2/4**	**6.10**	**0.0360**	**4/8**	**8.33**	**0.0007**
Structural constituent of cytoskeleton	**5/53**	**1.80**	**0.1996**	**9/31**	**3.54**	**0.0006**	3/39	1.28	0.5061

Table IB. *Ontological mapping of genes down-regulated by hypoxia.*

Gene Ontology	MDA-MB-468			MCF-7			T47D		
	# genes	abundance	*p*-value	# genes	abundance	*p*-value	# genes	abundance	*p*-value
Biological process									
Glycolysis	1/26	0.91	1.0	0/14	na	0.1458	2/23	0.91	1.0
RNA binding	**4/46**	**2.05**	**0.1294**	39/177	1.41	0.0177	**47/197**	**2.49**	**<0.0001**
mRNA splicing	**5/48**	**2.45**	**0.0511**	**12/38**	**2.02**	**0.0116**	**15/45**	**3.47**	**<0.0001**
rRNA processing	**2/8**	**5.89**	**0.0424**	**3/6**	**3.20**	**0.0526**	**5/8**	**6.51**	**0.0003**
Polyamine metabolism	**3/7**	**10.09**	**0.0023**	1/7	0.91	1.0	**1/6**	**1.74**	0.4545
Nucleotide metabolism	**5/61**	**1.93**	**0.1848**	8/38	1.35	0.3658	**10/46**	**2.26**	**0.0101**
Nitrogen metabolism	1/19	1.24	0.5623	**6/10**	**3.83**	**0.0017**	2/14	1.49	0.6375
Amino acid metabolism	3/91	0.78	1.0	**17/55**	**1.98**	**0.0039**	8/69	1.21	0.5343
Protein folding	2/50	0.94	1.0	**13/38**	**2.19**	**0.0050**	**11/43**	**2.67**	**0.0018**
Protein binding	20/476	0.99	1.0	43/262	1.05	0.7176	**6/10**	**6.25**	**0.0001**
Phosphate metabolism	10/294	0.80	0.5488	19/143	0.85	0.4765	19/347	*0.57*	*0.0046*
Translational initiation	1/25	0.94	1.0	5/24	1.33	0.4090	*7/187*	*0.39*	*0.0029*
Ribosome biogenesis & assembly	**2/10**	**4.71**	**0.0645**	**4/7**	**3.65**	**0.0139**	**7/24**	**3.04**	**0.0057**
Regulation of cell cycle	**23/190**	**2.85**	**<0.0001**	26/120	1.38	0.0701	16/157	1.06	0.7803
Cell cycle checkpoint	1/21	1.12	0.5989	**5/14**	**2.28**	**0.0542**	0/21	na	0.2548

Gene Ontology	MDA-MB-468			MCF-7			T47D		
	# genes	abundance	*p*-value	# genes	abundance	*p*-value	# genes	abundance	*p*-value
Mitotic cell cycle	**25/167**	**3.53**	**<0.0001**	26/115	1.44	0.0468	22/153	1.50	0.0473
S-phase of mitotic cell cycle	**7/75**	**2.20**	**0.0386**	11/49	1.43	0.2293	**14/63**	**2.32**	**0.002**
M-phase	**13/76**	**4.03**	**<0.0001**	**13/52**	**1.60**	**0.0790**	6/73	0.86	**0.8413**
DNA replication & chromosome cycle	**13/90**	**3.40**	**<0.0001**	13/58	1.43	0.1459	**19/78**	**2.54**	**<0.0001**
Cytokinesis	**11/48**	**5.40**	**<0.0001**	5/36	0.89	1.0	3/45	0.69	0.7966
Signal transduction	*19/749*	*0.60*	*0.0089*	42/349	0.77	*0.0449*	*28/462*	*0.63*	*0.0043*
Transport	*12/566*	*0.50*	*0.0048*	39/326	0.76	*0.0479*	*28/407*	*0.72*	*0.0455*
Development	*14/502*	*0.66*	*0.0970*	29/213	0.87	*0.4287*	*14/270*	*0.54*	*0.0088*
Molecular function									
CDK activity	**3/11**	**6.42**	**0.0097**	1/10	0.64	1.0	**2/11**	**1.89**	**0.2855**
Nucleic acid binding	53/892	1.40	0.0063	91/540	1.08	0.3775	86/672	1.33	0.0021
Biotin binding	**1/5**	**4.71**	**0.1951**	**3/3**	**6.39**	**0.0038**	0/4	na	1.0
Solute:cation symporter activity	0/12	na	1.0	**4/6**	**4.26**	**0.0068**	1/7	1.49	0.5070
Chaperone activity	3/68	1.04	0.7648	**15/50**	**1.92**	**0.0091**	**10/60**	**1.74**	**0.0731**
Endopeptidase activity	*2/83*	*0.57*	*0.5836*	7/35	1.28	0.4803	**12/49**	**2.55**	**0.0017**
RNA helicase activity	0/14	na	1.0	**5/12**	**2.66**	**0.0280**	**6/15**	**4.17**	**0.0018**
Transmembrane receptor activity	*5/218*	*0.54*	*0.1672*	10/72	0.89	0.8688	*3/112*	*0.28*	*0.0079*
Metal ion binding	14/389	0.85	0.5972	29/200	0.93	0.6845	*14/267*	*0.55*	*0.0087*
Cellular component									
Proteasome complex	0/22	na	1.0	**4/17**	**1.50**	**0.3243**	**10/21**	**4.96**	**<0.0001**
26S proteasome	1/30	0.78	1.0	**10/24**	**2.66**	**0.0019**	**13/29**	**4.67**	**<0.0001**
Cytoskeleton	*10/373*	*0.63*	*0.1373*	23/212	0.69	0.0465	*14/279*	*0.52*	*0.0051*
Nucleus	**74/1031**	**1.69**	**<0.0001**	115/629	1.17	0.0383	102/787	1.35	0.0003
Chromosome	**7/52**	**3.17**	**0.0059**	2/30	0.43	*0.2128*	6/43	1.45	0.2976
Ribonucleoprotein complex	6/131	1.08	0.8241	16/114	0.90	0.6931	**25/122**	**2.13**	**0.0002**
Mitochondrion	11/274	0.95	1.0	34/181	1.20	0.2400	**40/229**	**1.82**	**0.0001**

Biological processes, cellular components and molecular functions affected by hypoxia in the three breast cancer cell lines MDA-MB-468, MCF-7 and T47D annotated according to Gene Ontology terms (14). A. Genes up-regulated by hypoxia. B. Genes down-regulated by hypoxia. # genes=number of genes with altered gene expression/number of well measured genes in experiment; abundance=fold level of expected change; *p*-value=significance of number of altered genes. Values in bold represent an over-abundance of genes in the pathway, italicised values represent a decreased abundance of genes in the pathway, and roman values represent no change in abundance over the expected. na, not applicable.

Effects on the cytoskeleton were mainly found among genes involved in intermediate filament structures in the three cell lines, but not actin or microtubule cytoskeleton associated genes (Table IA). In addition, considerable variation in other functional and structural effects was seen between the different cell lines. Specifically, genes involved in sterol biosynthesis were over-represented among the up-regulated genes in T47D (Table IA). There was also a unique acute phase immune response in MDA-MB-468 cells following hypoxia, as well as more prominent effects on genes involved in cell cycle regulation and cytokinesis (Table I). Taken together, this may reflect how different cell cycle and other defects are overcome as the cells adapt to environmental stress in different ways.

Recent clinical studies suggest that hypoxia enhances malignant progression and increases tumour aggressiveness through promotion of genomic changes and clonal selection. An interesting study by Woelfle *et al.* was published recently where a molecular signature of 86 genes associated with bone marrow micrometastasis (BM) in breast cancer was identified (25). The major functional categories of primary tumours with BM genes found in that study included extracellular matrix remodelling, cytoskeleton plasticity, and the IFN and HIF-1α signal transduction pathways. Interestingly, although it has been suggested that HIF-1α is not regulated on the transcriptional level, they detected higher levels of HIF-1α mRNA in BM-positive compared to -negative tumours (25). The expression of lactate dehydrogenase was also higher in BM-positive compared to -negative tumours, and was induced by hypoxia in our study. Taken together, these studies suggest that the presence of hypoxia and the dysregulation of the HIF-1α signal

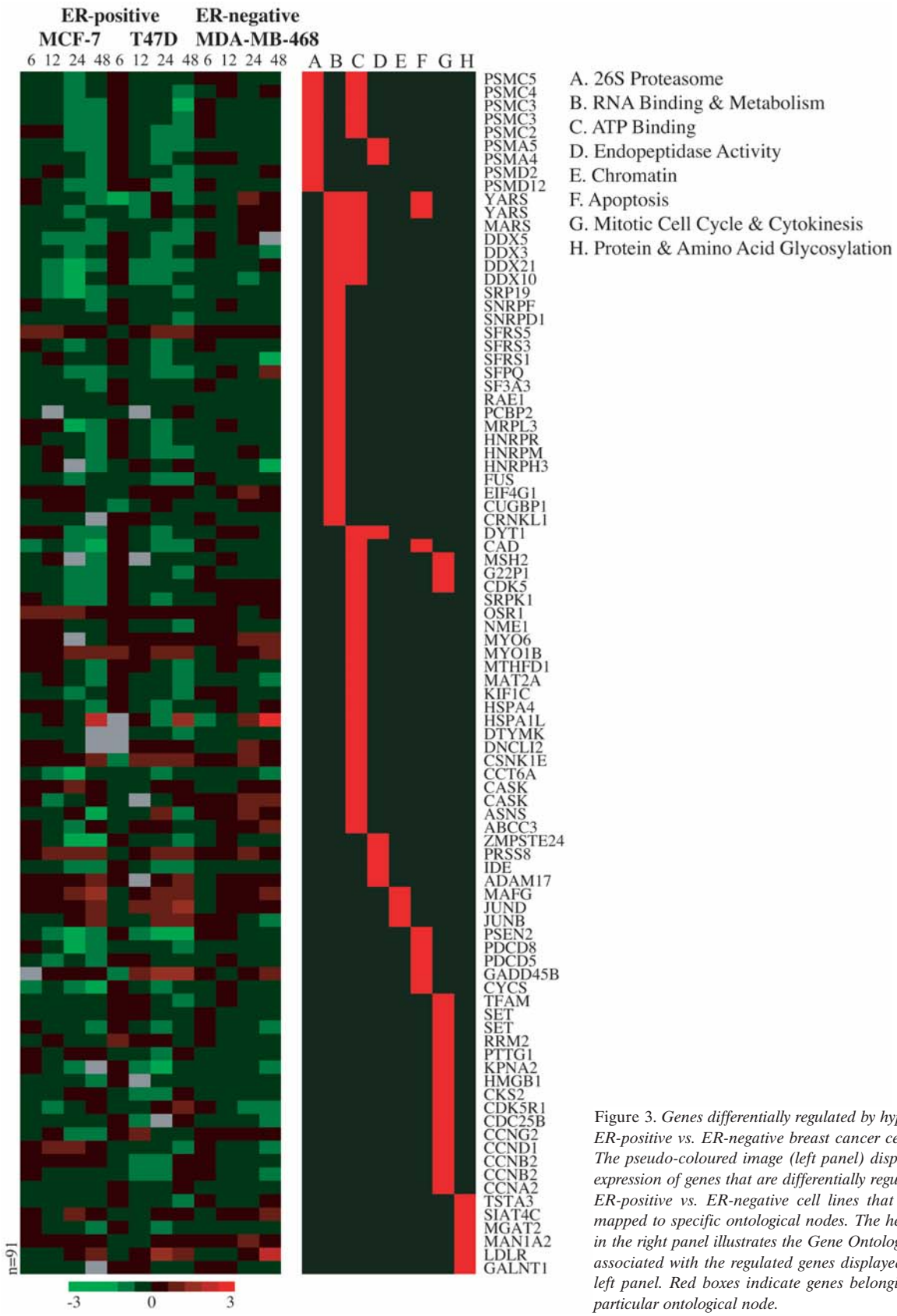

Figure 3. *Genes differentially regulated by hypoxia in ER-positive vs. ER-negative breast cancer cell lines. The pseudo-coloured image (left panel) displays the expression of genes that are differentially regulated in ER-positive vs. ER-negative cell lines that can be mapped to specific ontological nodes. The heat map in the right panel illustrates the Gene Ontology terms associated with the regulated genes displayed in the left panel. Red boxes indicate genes belonging to a particular ontological node.*

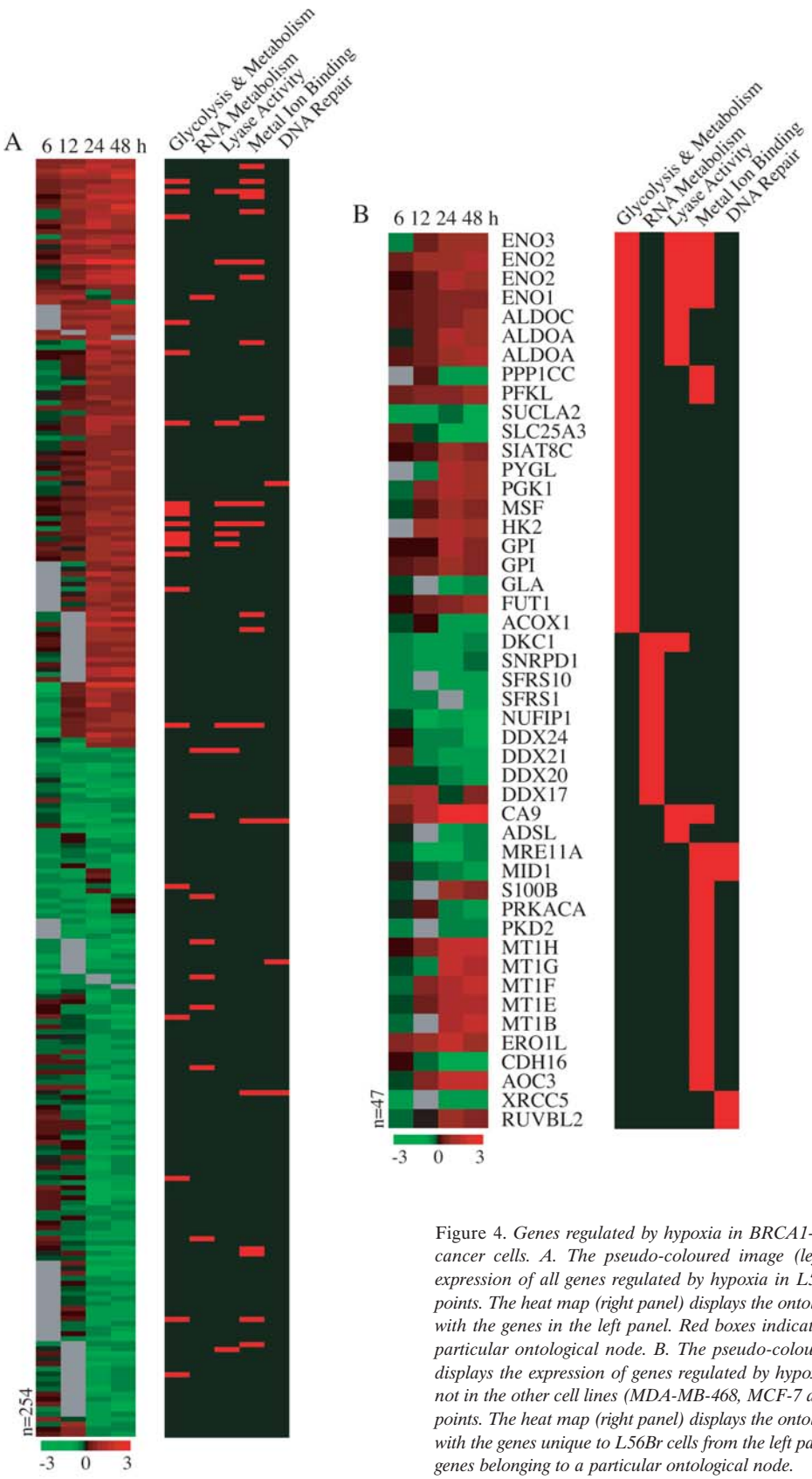

Figure 4. *Genes regulated by hypoxia in BRCA1-mutated L56Br breast cancer cells. A. The pseudo-coloured image (left panel) displays the expression of all genes regulated by hypoxia in L56Br cells at four time points. The heat map (right panel) displays the ontological terms associated with the genes in the left panel. Red boxes indicate genes belonging to a particular ontological node. B. The pseudo-coloured image (left panel) displays the expression of genes regulated by hypoxia in L56Br cells, but not in the other cell lines (MDA-MB-468, MCF-7 and T47D) at four time points. The heat map (right panel) displays the ontological terms associated with the genes unique to L56Br cells from the left panel. Red boxes indicate genes belonging to a particular ontological node.*

Table II. *HIF-1 binding site sequences in regulated vs. non-regulated genes.*

# genes	MDA-MB-468		MCF-7		T47D		L56Br	
	regulated	non-regulated	regulated	non-regulated	regulated	non-regulated	regulated	non-regulated
With motif	275	2684	417	1226	310	1711	100	1418
Without motif	471	5120	647	2322	547	3238	118	2567
OR (*p*-value)	1.11 (0.1838)		**1.22 (0.0061)**		1.07 (0.37)		**1.53 (0.0023)**	

A global search for potential hypoxia responsive elements (HRE) in the upstream region (10,000 basepairs upstream and 1,000 basepairs downstream) of hypoxia-regulated and non-regulated genes was performed. A conservative motif of eight nucleotides was used in the analysis (5'-G/C/T-ACGTGC-G/T-3'). OR=odds ratio; *p*-value=significance of OR (Fisher's exact test). Values in bold represent a significant OR.

transduction pathway in primary tumours may be an early risk indicator for micrometastases in breast cancer. It has recently been suggested that hypoxia promotes dedifferentiation in breast cancer, thereby contributing to tumour progression (19). The up-regulation of *NOTCH2*, which has been implicated in differentiation in many tissue types, in the breast cancer cell lines in our study is interesting in this context and warrants further investigation. However, the down-regulation of several cytokeratins, both luminal and basal, in the cell lines subjected to hypoxia in the present study as well as in the primary tumours of BM-positive breast cancers in the study by Woelfle and colleagues may indicate a role for these genes in tissue maintenance and/or metastasis suppression, rather than in the process of differentiation.

Low oxygen levels may directly or indirectly influence response to ionising radiation and chemotherapy. Down-regulation of apoptosis-related genes, as well as cell cycle perturbations caused by hypoxia, may in part explain the acquired resistance to some chemotherapeutic drugs that is well documented. Moreover, down-regulation of genes involved in DNA repair mechanisms (double strand break repair and non-recombinational repair) in the *BRCA1*-deficient cell line L56Br may reflect the crucial role played by the BRCA1 protein in instances of DNA damage, such as during hypoxia. This, in turn, may explain why tumours caused by *BRCA1* mutations often display considerable genetic alterations, especially when taking into consideration the fact that these tumours often grow rapidly and, therefore, neovascularisation may be greatly perturbed. In the context of radiation resistance, metallothioneins have been postulated to play a role in zinc and copper homeostasis and to protect against reactive oxygen species. Interestingly, hypoxia caused induction of several metallothioneins (*MT1B, E, F, G* and *H*) in L56Br cells in the present study. These findings may suggest that mutated *BRCA1* in breast cancer cells exposed to decreased oxygen pressure allows for an increased level of transformation, while the level of proliferation is not affected to any great extent.

Through binding to HREs, HIF-1 regulates the expression of a number of genes encoding glycolytic and angiogenic proteins, thereby supporting cell survival during low oxygen pressure. It is believed that the presence of the HIF-1 binding HRE motif in the promoter of genes indicates HIF-1 responsiveness in these genes. Indeed, in a recent study by Leonard and colleagues, HRE consensus motifs were widely distributed in the promoters of hypoxia-dependent genes in renal epithelial cells exposed to hypoxia (26). However, they did not perform an equivalent analysis of the presence or absence of such motifs in genes not responsive to hypoxia. Our findings illustrate a significant association between the presence of the HIF-1 binding consensus motif and hypoxia-responsive genes in two of four cell lines investigated; but, interestingly, the motif was also present among several genes found not to respond to low oxygen pressure (Table II). Moreover, when a less stringent motif was used in the analysis (5'-ACGTG-3', used in the MatInspector™ software utilised *e.g.* in the study by Leonard *et al.* (26)), there was no significant difference in the presence of the motif between hypoxia-responsive and non-responsive genes. The large number of false-positives indicates that the consensus motif for identifying HIF-1 binding sites among potential hypoxia-responsive genes needs to be complemented with functional studies to identify true HREs. It is possible that other factors are needed for an HRE to be functional, which are not present among the non-responsive genes displaying the HIF-1 binding motif in our study.

Tumour hypoxia is known to enhance malignant progression and increase aggressive behaviour of tumour cells. Hypoxia promotes genomic instability, thereby contributing to tumour cell heterogeneity and the potential for clonal selection of more malignant subclones (27).

Tumour hypoxia, reflected as over-expression of HIF-1α, has recently been identified as an independent prognostic marker in breast cancer (20, 21) and has been found to be a prognostic factor for overall as well as disease-free survival in various solid tumours (4). It is well documented that tumour hypoxia causes a malignant tumour phenotype and increases the risk for metastasis, as well as conferring resistance to radiation and some chemotherapeutic drugs, leading to treatment failure. Moreover, recent findings have shown that hypoxia can down-regulate expression of the ER, thereby potentially interfering with the response of ER- positive tumours to anti-oestrogen (*e.g.* tamoxifen) treatment (22-24). This, in turn, may explain the acquired resistance of many breast tumours to hormone therapy.

The profound transcriptional changes induced by hypoxia illustrated in the present study help explain how tumour cells adapt to a hypoxic environment and circumvent the growth retardation normally caused by hypoxia. Based on the dependence of tumour growth on angiogenesis, and the notion that oxygen starvation of tumours would inhibit tumour growth and progression, the concept of anti-angiogenic therapy for the treatment of malignant tumours has emerged (28). Whereas promising effects of anti-angiogenic drugs have been documented in animal models (*e.g.* the angiogenesis inhibitor endostatin (29)), these drugs have not had the desired impact on tumour treatment in clinical trials (30).

Our findings emphasise that tumour cells may adapt to decreased levels of O_2 in different ways, depending on the genetic background and phenotypic characteristics of the tumour cells. Down-regulation of apoptosis-related genes, as well as cell cycle perturbations caused by hypoxia may, in part, explain the acquired resistance to some chemotherapeutic drugs that is well documented. Moreover, down-regulation of genes involved in DNA repair mechanisms in *BRCA1*-deficient cells may reflect the crucial role played by BRCA1 in instances of DNA damage, such as during hypoxia. This may suggest that mutated *BRCA1* in breast cancer cells exposed to decreased oxygen levels allows for an increased level of transformation, while the level of proliferation is not affected to any great extent. The profound and differential transcriptional changes induced by hypoxia illustrated in the present study help explain how tumour cells adapt to a hypoxic environment and circumvent the growth retardation normally caused by hypoxia, and may shed further light on the effects of hypoxia on tumour evolution and treatment response.

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