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TIME-DEPENDENT TRANSCRIPTIONAL CHANGES IN A BREAST CANCER CELL LINE CAUSED BY HYPOXIA

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Breast cancer is the most common form of malignant disease affecting women in the Western World. Nearly 50% of locally advanced breast cancers exhibit areas of hypoxia which is associated with poor prognosis. The aim of this project was to investigate the time dependency of transcriptional changes due to *in vitro* hypoxia treatment of a breast cancer cell line. cDNA microarrays containing 27,000 clones were used to analyze gene expression patterns at different time points of hypoxia treatment. A time series analysis of the microarray data was performed to reveal genes exhibiting specific patterns of expression. Hypoxia treatment affected the expression of genes involved in glucose metabolism, apoptosis, cell cycle progression and angiogenesis. Notably, the genes involved in glucose metabolism were induced earlier than cell cycle regulatory genes. Furthermore, Lysyl Oxidase-like 2, a gene recently implicated in breast cancer invasion, was strongly upregulated by the hypoxic treatment. This may be one of the several mechanisms by which hypoxia affects the malignant progression of tumors.

Populärvetenskaplig sammanfattning:

Hur undviker en tumör att kväva sig själv?

Cellerna är kroppens byggstenar. Olika celler har olika funktioner och cellerna samarbetar i balans med varandra för att vi ska få en fungerande kropp. Vid cancer har den normala funktionen hos celler rubbats och cancercellerna fungerar inte längre i balans med resten av kroppens celler utan blir fler och fler tills de bildar en tumör. Efter hand kan vissa cancerceller lämna tumören och vandra iväg till andra områden i kroppen där de bildar en ny tumör, en s k metastas. Om en tumör bildar metastaser är det mycket svårt att bota sjukdomen som då ofta blir dödlig. När en tumör blir större och större bildas områden i tumörmassan med brist på syre eftersom blodkärlen runt tumören inte räcker till för att ge alla celler syre. Många tumörceller slutar då att växa och dör, men vissa anpassar sig till den låga syrehalten och blir tyvärr farligare för kroppen än de celler som inte har syrebrist. Dessa celler signalerar till sin omgivning att de vill ha mer syre och får omgivningen att bilda nya blodkärl. Dessutom försöker dessa tumörceller rymma från tumören till en annan plats i kroppen där de kan bilda metastaser. Därför är det viktigt att hitta en behandling som hejdar de förändringar som sker vid syrebrist i en tumör.

Jag ville undersöka vad som mer exakt händer med tumörceller när vi utsätter dem för syrebrist. När förändras cellerna och hur blir de farligare? Sker vissa förändringar tidigare än andra? Den cancerform jag undersökte var bröstcancer. Det är en av de vanligaste cancerformer som drabbar kvinnor och ungefär en av tio svenskor kommer att drabbas av bröstcancer under sin livstid. För att undersöka hur bröst cancer-celler påverkas av låg syrehalt utsatte vi våra celler för syrebrist under olika tidslängder. Det visade sig att vissa förändringar sker tidigare än andra, till exempel förändras cellens sätt att bilda energi tidigt medan cellens kontroll av sin egen tillväxt förändras senare. Dessutom såg vi förändringar som pekar på att cellerna lättare kan vandra iväg och bilda nya tumörer. Resultaten har ökat vår förståelse för vad som får tumörceller att anpassa sig till syrebrist och kan kanske hjälpa oss att hitta nya, bättre behandlingsmetoder mot cancer i framtiden.

INTRODUCTION

Cancer cells suffer from defects in their regulation of cell proliferation and homeostasis. Tumorigenesis is considered to be a multistep process and each step represents genetic alterations that drive the transformation of normal cells into highly malignant derivatives.
Hanahan and Weinberg [1] have proposed six essential alterations in cell physiology that control malignant growth;

- Self-sufficiency in growth signals
- Insensitivity to antigrowth signals
- Evasion of apoptosis
- Limitless replicative potential
- Sustained angiogenesis
- Tissue invasion and metastasis

Several of these alterations involve control of the cell cycle. The cell cycle is the fundamental means by which cells duplicate their contents and then divide into two. The cell cycle is divided into four phases:

G1 phase (integrating mitogenic and growth inhibitory signals and making the decision to proceed, pause or exit the cell cycle)
S phase (DNA synthesis)
G2 phase (preparing for the division process)
M (mitosis, cell division)

The cell cycle is regulated by the synthesis and destruction of cyclins that associate with and activate cyclin-dependent kinases (CDKs) [2]. To control the passage through the cell cycle there are different checkpoints which may inhibit the cell cycle progression due to *e.g.* a lack of growth factors, or if the cell contains damaged DNA. The control over proliferation is disturbed in cancer cells and several genes which are mutated in cancer cells are involved in the progression of the cell cycle. For a summary of the cell cycle, see *Figure 1*.

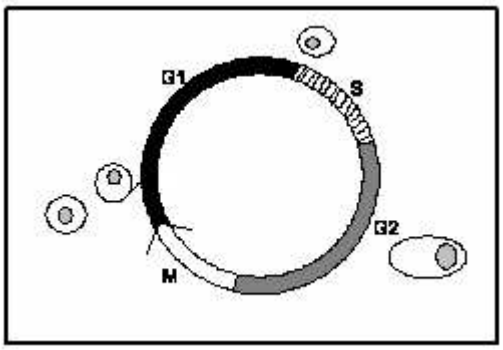

Figure 1. **A schematic figure of the cell cycle phases.**

During Gap 1 phase the cell receives signals to make the decision to proceed, pause or exit the cell cycle.
During S phase, the cell replicates its DNA, In Gap 2 phase, the cell is increasing its volume and making a second copy of all organelles for the new cell. During Mitosis (M) the cell is divided into 2 daughter cells.

S: S phase, G2: Gap 2 phase, M: Mitosis, G1: Gap 1 phase.

The traditional approach to improve understanding of the molecular basis of cancer has been to study the behavior of individual genes. It is however well known that multiple interconnected pathways are involved. The development of large-scale gene expression profiling tools has made it possible to investigate the global variation in transcriptional expression profiles, thus providing a more comprehensive overview of the complex network of underlying genetic defects leading to the development of cancer.

BREAST CANCER

Breast cancer is the most common form of cancer affecting women in Europe, North America and other Western countries. Women in the Western world are estimated to have a lifetime risk of approximately 10% to develop breast cancer (described by Hedenfalk, 2002 [3]). As with other solid tumors, breast cancer development is considered to be a multi-step process, accumulating genetic and epigenetic alterations which lead to increased aggressiveness.

Risk factors for breast cancer

Approximately 5-10% of all breast cancer cases exhibit a familial pattern of incidence. *BRCA1* and *BRCA2* are two major breast cancer susceptibility genes, and germ line mutations in these genes account for 15-20% of breast cancer that clusters in families and less than 5% of overall breast cancer [4]. Female mutation carriers of *BRCA1* have a lifetime breast cancer risk of 60-80%. The corresponding lifetime risk for *BRCA2* mutation carriers is estimated to be 60-85% (reviewed by Nathanson *et al*., 2001 [4]) .

Most breast cancers are however sporadic in origin and little is known about the underlying genetic defects. Other risk factors for breast cancer reported in the literature include early menarche, late menopause, late first birth, nulliparity, obesity, alcohol consumption, hormone replacement therapy and excessive radiation exposure [5].

Estrogen, progesterone and breast cancer

Estrogen and progesterone and their receptors (ER and PR) play an important role in the development and function of the mammary gland and appear to also play a significant role in the development, progression, treatment and outcome of breast cancer [6]. One-third of all breast cancers lack ER and PR expression and this is associated with less differentiated tumors and poorer clinical outcome. ER is a target for endocrine therapy by *e.g.* tamoxifen (an anti-estrogen). However, only ~50% of patients with ER-positive (ER+) tumors and ~75% of patients with ER+ and PR+ tumors will respond to endocrine therapy [7]. There are many possible mechanisms for this lack of response to endocrine therapy. As an example, there are indications that ER may be heterogeneously expressed in ER+ tumors and that the expression may be downregulated by *e.g.* hypoxia which interferes with the activity of tamoxifen, as discussed below.

HYPOXIA

Tissue hypoxia results from deregulated proliferation of malignant cells and an insufficient supply of oxygen (O_2) due to structurally and functionally disturbed microcirculation. Solid human tumors frequently contain regions that are deficient in oxygen. Nearly 50% of locally advanced breast cancers exhibit hypoxic and/or anoxic areas [8].

HYPOXIA AS A PROGNOSTIC FACTOR

Tumor hypoxia does not depend on clinical tumor size, clinical stage, histological type, grade or extent of necrosis, and is therefore considered to be an independent prognostic factor for overall and disease-free survival for *e.g.* squamos cell carcinomas of the uterine cervix and of the head and neck [9]. Hypoxia is associated with a poor prognosis as it has been shown to reduce sensitivity to conventional treatment and increase malignant progression [10].

Treatment resistance

There are multiple mechanisms by which hypoxia may confer treatment resistance, such as a reduced generation of free radicals (due to lack of oxygen) and inhibition of cell proliferation. The drug delivery to hypoxic areas may also be challenged since tumor hypoxia itself arises from insufficient vasculature. Furthermore, hypoxia may downregulate the expression of the estrogen receptor [11] and may therefore contribute to the acquired resistance to hormonal therapy in breast cancer.

- The radiation dose required to achieve the same biological effect is 2.8-3 times higher in the absence of oxygen than in the presence of normal levels of oxygen [9].

- Several cytotoxic drugs have been shown to be oxygen dependent [9].

Malignant progression

The progression of many types of cancer can be divided into distinct stages in which tumors gradually develop aggressive phenotypic traits. The final stage of the progression is the development of cell variants showing invasive growth in surrounding normal tissues and metastatic spread to distant organs [12]. *Figure 2* illustrates the role of hypoxia in malignant progression.

Hypoxia has been implicated in promoting metastasis in several studies [13]. Hypoxia may promote metastasis in different ways, not only by inducing the expression of genes involved in metastasis but also by providing selection pressure for a more aggressive phenotype. Hypoxia has also been shown to induce point mutations, deletions and gene amplifications which can have an impact on the clonal selection of more aggressive cells [12].

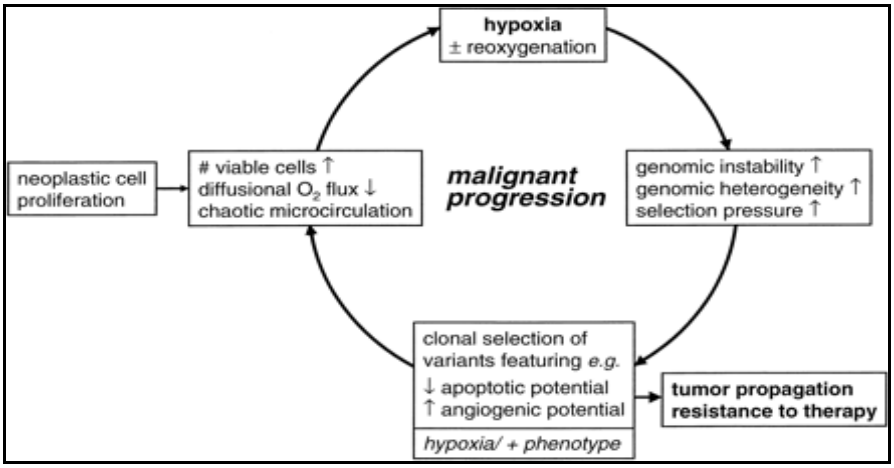

Figure 2. **The importance of hypoxia in malignant progression.**

Hypoxia is a result of the deregulated proliferation and the insufficient circulation. Hypoxia causes genomic instability and an increased selection pressure which leads to the clonal selection of more aggressive cells. Reprinted from Höckel and Vaupel (2001) [14].

To illustrate hypoxia-mediated selection of tumor cells with a reduced apoptotic potential Graeber and colleagues performed an experiment where embryonic fibroblasts derived from wild-type and p53-deficient mice were investigated. They mixed $p53^{+/+}$ (wt) cells with $p53^{-/-}$ cells in a 1000:1 ratio. After submitting the cells to multiple rounds of hypoxia and aerobic recovery the $p53^{-/-}$ cells had overtaken the $p53^{+/+}$ cells [15].

THE CELLULAR RESPONSE TO HYPOXIA

Cellular responses to hypoxia provide the essential compensatory mechanisms to increase the delivery of oxygen and nutrients, while removing the waste products of metabolism.

HYPOXIA-INDUCIBLE FACTOR 1 (HIF-1)

HIF-1 (Hypoxia-inducible factor 1) is the master regulator of oxygen homeostasis [16]. HIF-1 is a transcription factor composed of a 120 kDa HIF-1α and a 91-94 kDa HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator: ARNT) subunit. Both subunits contain a basic helix-loop-helix (bHLH) DNA binding domain and a Per/ARNT/Sim (PAS) domain (*Figure 3*). HIF-1β is a constitutive nuclear protein while HIF-1α is inducibly expressed.

HIF-1α epression is mainly regulated at the level of protein synthesis and degradation. Hypoxia has been shown to only have a transient effect on HIF-1α mRNA levels [17], increasing mRNA levels after 30-60 minutes of hypoxic treatment but returning to baseline levels after 4 hours.

Under normoxic conditions the oxygen-dependent degradation (ODD) domain of the HIF-1α protein is hydroxylated on proline residues 402 and 564 by HIF-1α prolyl-hydroxylases which require oxygen, ferrous iron and 2-oxoglutarate for activity [18]. This hydroxylation allows HIF-1α binding to the tumor suppressor Von Hippel-Lindau (VHL) which causes HIF-1α to be ubiquitinated and degraded by the proteasome. The carboxy-terminal *trans*-activation (TA) domain is hydroxylated by an oxygen-dependent asparaginyl hydroxylase which blocks the interaction with the CBP/p300 coactivator (*Figure 3*).

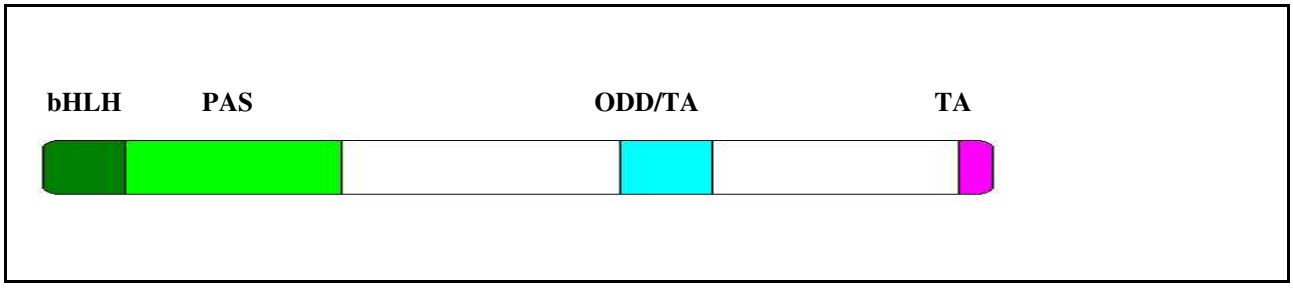

Figure 3. **Schematic illustration of HIF-1α's functional domains.**
HIF-1α contains a basic helix-loop-helix (bHLH) domain for DNA binding and a Per/ARNT/Sim (PAS) domain for interaction with HIF-1β. The oxygen-dependent degradation (ODD) domain is hydroxylated during normoxic conditions at two proline residues yielding binding to VHL and subsequent degradation. The carboxy-terminal *trans*-activation (TA) domain interacts with coactivator CBP/p300 during hypoxic conditions. During normoxic conditions it is subjected to asparaginyl hydroxylation which blocks the interaction with CBP/p300.

Under hypoxic conditions HIF-1α is not hydroxylated and therefore escapes the recognition by VHL and subsequent proteasome degradation. Stabilized HIF-1α is translocated to the nucleus where it interacts with HIF-1β (ARNT) and other co-factors such as CBP/300 and the DNA polymerase II (Pol II) complex. HIF-1, together with its cofactors, binds to genes containing hypoxia responsive element (HREs) and activates transcription (for a summary see *Figure 4*). HIF-1α is not only regulated by the O_2 concentration of the cell but has also been shown to be stabilized during normoxic conditions by many growth factors and cytokines [19], *e.g.* insulin, insulin-like

growth factors 1 and 2 (IGF-1, IGF-2), interleukin-1β (IL-1β), epidermal growth factor (EGF), fibroblast growth factor 2 (FGF-2), tumor necrosis factor α (TNF-α), platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β). Most of these growth factors might stabilize HIF-1α via common cellular kinase pathways (*Figure 4*).

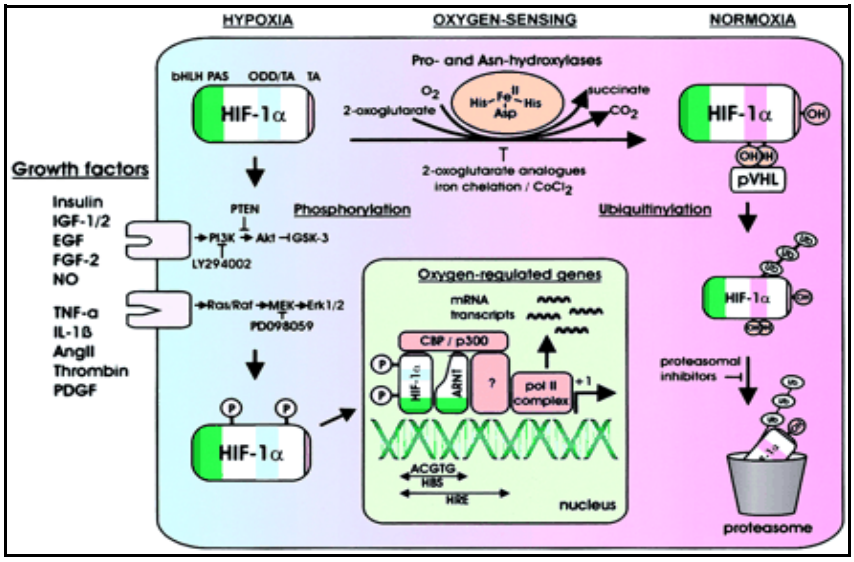

***Figure 4.* Schematic model of oxygen sensing and HIF-1 regulation.**

Under hypoxic conditions HIF-1α is stabilized, enters the nucleus and activates gene expression. Under normoxic conditions HIF-1α is hydroxylated, targeting HIF-1α for pVHL-mediated proteolytic destruction.
bHLH; basic helix-loop-helix, HBS; HIF-1 binding site, HRE; hypoxia response element, ODD; oxygen-dependent degradation, PAS;PerAhR/ARNT-SIM, TA; *trans*-activation, UB; ubiquitin.
Reprinted from Wenger 2002 [19].

HIF-1 TARGET GENES

Over 40 HIF-1 target genes have been identified [16] which encode proteins that play key roles in several biological processes, including angiogenesis/vascular remodeling, glucose transport, glycolysis, pH regulation, cell adhesion, coagulation, iron transport, cell proliferation and apoptosis (Box 1).

Box 1. Examples of HIF-1 target genes (See ref. [16]).

Angiogenesis and vascular remodeling

Adrenomedullin | Endothelin-1 | Vascular endothelial growth factor (VEGF)

Glycolysis and glucose uptake

Aldolase A and C | Enolase 1 | Glucose transporter 1 and 3 (GLUT-1 and -3) | Hexokinase 1 and 2 | Lactate dehydrogenase A | Glyceraldehyde 3- phsophate dehydrogenase (G3PD)| Phosphofruktokinase L (PFKL) | Phosphoglycerate kinase 1 | Pyruvate Kinase M (PKM)

Iron transport and pH regulation

Transferrin | Transferrin receptor | Carbonic anhydrase 9 (CA IX)

Cell proliferation and apoptosis

Insulin-like growth factor 2 (IGF-2) | NIP3/NIX

Cell adhesion and coagulation

Collagen type V, α1 | prolyl-4 hydroxylase α(I)

Glucose metabolism

The primary pathway for generation of ATP in eukaryotic cells under normoxic conditions involves the oxygen-dependent tricarboxylic acid (TCA) cycle. Tumor cells are characterized by an increase in the oxygen-independent glycolytic metabolism, even in the presence of high O_2 concentrations, a phenomenon known as "the Warburg effect" [20]. Glucose transport into tumor cells is also increased. Hypoxia has been shown to increase the glycolytic pathway and glucose transport into the cells, as HIF-1 has been shown to mediate transcriptional activation of several steps in the glycolysis, and also the uptake of glucose (Box 1). The end product of glycolysis is lactate. A correlation between lactate production and metastasis has been reported for uterine cervix and head and neck tumors [21].

Angiogenesis

Tumor growth beyond a volume of 1-2 mm^3 has been shown to require neovascularization [22]. Angiogenesis is the development of new blood vessels from the existing vasculature.
Tumor angiogenesis occurs as a result of increased expression of angiogenic factors and decreased expression of anti-angiogenic factors. Vascular endothelial growth factor (VEGF) is a major inducer of angiogenesis and is known to be upregulated by hypoxia (Box 1). There is a strong correlation between VEGF expression and blood vessel density and clinical outcome in several tumor types [23]. Other angiogenic factors are also induced by hypoxia, *e.g.* platelet derived growth factor (PDGF), endothelin, insulin-like growth factor 2 (IGF-2) and epidermal growth factor (EGF) [24].

Apoptosis

Apoptosis, or programmed cell death, is induced by hypoxia by several different pathways;

- HIF-1 activates Bcl-2 and nineteen-kilodalton interacting protein-3 (BNIP3) and Nip3-like protein X (NIX) expression (two pro-apoptotic proteins) in many tumor types, as well as in endothelial cells and macrophages [25].

- HIF-1 promotes p53-dependent apoptosis [26]. Dephosphorylated HIF-1 directly interacts with p53 and may promote apoptosis by stabilizing the p53 protein. p53 induces the expression of for example the pro-apoptotic protein Bax.

THE cDNA MICROARRAY TECHNOLOGY

The cDNA microarray technology allows for parallel analysis of the expression of thousands of genes, hence it is possible to investigate the global variation in transcriptional expression profiles [27]. *Figure 5* schematically illustrates the main principle for the cDNA microarray technology. cDNA arrays are produced by robotic spotting of PCR products of approximately 0.6-2.4 kb [28], representing specific genes, onto a matrix (typically a glass microscope slide). Total RNA is extracted from the samples of interest, reverse transcribed into cDNA and labeled with fluorochromes. Typically, a reference sample is labeled with a certain fluorochrome and an experimental sample with another. The labeled cDNA is pooled and allowed to co-hybridize to the microarray. The slide is then scanned at different wavelengths and the ratio of the two fluorescence intensities at each spot is calculated and represents the relative expression of that gene in the experimental sample compared to the reference.

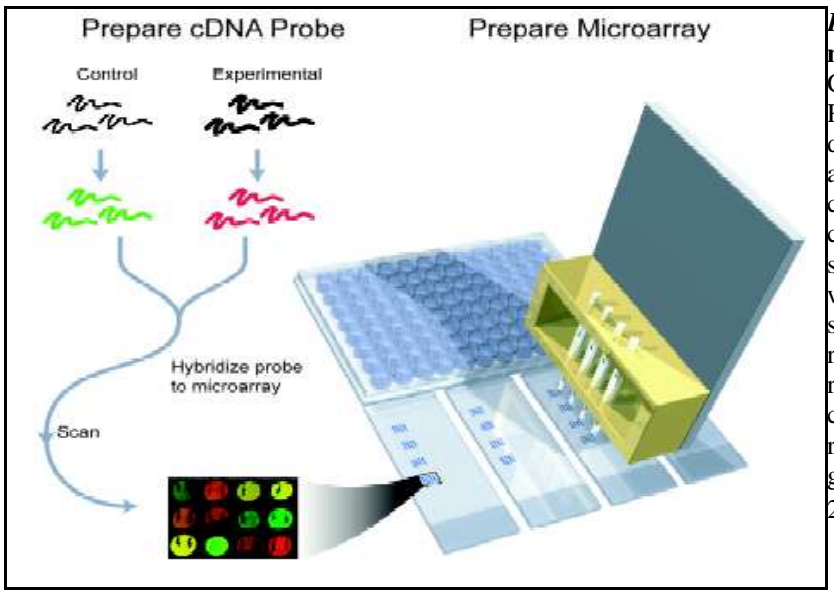

Figure 5. **Overview of the cDNA microarray technology.** Control (reference) and experimental RNA is reversibly transcribed and differentially labeled with fluorochromes and co-hybridized onto a matrix containing robotically printed cDNA clones representing individual genes. The slides are scanned at two different wavelengths and the images are superimposed, yielding an image of the ratio of the intensities where genes up-regulated in the experimental sample compared to the reference sample appear red and genes down-regulated appear green. Reprinted from Hedenfalk *et al.,* 2002 [29].

The goal of a microarray experiment is to measure the mRNA expression of thousands of genes in a sample. Further on, the expression profile of a certain experimental sample should be comparable to another experimental sample yielding the relative expression between for example a treated sample and an untreated sample. However, there are many sources of variation in a microarray experiment which need to be acknowledged and reduced if possible. To produce good quality microarray data there are many considerations [30]. Some of these are presented here:

Probes

The ideal microarray for global expression profiling contains a large number of sequence-validated probes in which each sequence shows minimal cross-hybridization to related sequences and therefore represents the expression of a certain gene and not its homologues. The arrangement of spots on the slide can impact the analysis of microarray data [31]. For example groups of spots printed by the same pins may lead to correlations among those spots. The position of the spots should be randomized to minimize correlations due to for example differences in background intensity.

Printing substrates

Spotted arrays are typically printed on glass slides. There are several different commercial slides available with different coating material, affecting the durability and the degree of background and signal intensity.

RNA labeling

Initial labeling protocols were based on “direct labeling” where the reverse transcription reaction incorporated fluorescently labeled nucleotides. The labeled nucleotides are difficult to incorporate using regular enzymes and there may be “dye biases” when rates of incorporation differ between dyes. To avoid these problems one may use an “indirect labeling” where an amino-allyl modified dUTP is used in the reverse transcription reaction. The dyes are coupled to the free amine group on the amino-allyl dUTP after the reverse transcription.

Reference RNA

cDNA arrays are generally used as a two-label system in which two RNA samples are separately labeled, mixed, and hybridized together to each array. A ratio of the intensity of hybridization of the two samples is calculated for each probe. The use of the same standard reference RNA for all arrays results in a standardization of the hybridization intensity with regard to variation in size and shape of corresponding spots on different arrays [32]. Therefore, more than two experimental samples are comparable.

Data analysis

The measured intensity for each spot represents the sample's expression level for that given gene. For this to be true different transformations need to be carried out [33]. A filtering step may be performed to eliminate questionable or low-quality measurements due to for example low spot intensity in comparison to the surrounding background (signal-to-noise ratio). Normalization of the data adjusts the individual hybridization intensities such that comparisons can be made. Normalization adjusts differences due to *e.g.* unequal amounts of starting RNA, differences in labeling efficiency and systematic biases in the measured expression levels. An example of a systematic bias is the indications that low intensity spots (weak hybridization) show greater variance in their measured ratios than high intensity spots. Locally weighted linear regression (lowess) analysis is a normalization method that may reduce some intensity-dependent effects.

Interpreting the data

Microarrays may generate tens of thousands of data points for each performed experiment. To make sense of the data, systematic methods are needed. There are several different tools available for the analysis and organization of large-scale expression data. The selection of an adequate method for analyzing the data depends on the nature of the performed experiment and which questions are to be answered.

Grouping together genes with similar patterns of expression is a natural interest in several experiments. This is frequently performed by using agglomerative hierarchical clustering methods where all data instances start in their own clusters, and the two most similar clusters are merged. This process of merging is repeated until a single cluster remains. Relationships among genes are frequently represented by a tree whose branch lengths reflect the degree of similarity between the objects.

FLOW CYTOMETRY

Flow cytometry allows for the measurement of physical and chemical characteristics of cells as they travel in suspension, one by one, past a sensing point. Most modern cytometers use a laser as a light source and collection lenses placed in front of the light source and at right angles of the light source. By computerized translation of the signals to data one may measure for example cell size, shape and any cell component that can be detected by a fluorescent compound [34].

CELL CYCLE ANALYSIS

To measure the abundance of cells in the different stages of the cell cycle one may stain the DNA of the cells with propidium iodide (PI). PI intercalates double-stranded DNA and upon binding the

fluorescence is enhanced ~20- to ~30-fold and its fluorescence maximum is shifted ~30-40 nm to the red. Since PI also binds to RNA one needs to treat the cells with RNase together with PI to distinguish between RNA- and DNA-binding. One important aspect of DNA analysis is to exclude cell doublets since these may interfere with the analysis of single cells. In order to analyze the different stages of the cell cycle it is general praxis to create a DNA histogram (see *Figure 6*) and gate the features corresponding to the cell cycle stages (either subjectively or mathematically), yielding a percentage of cells in each phase.

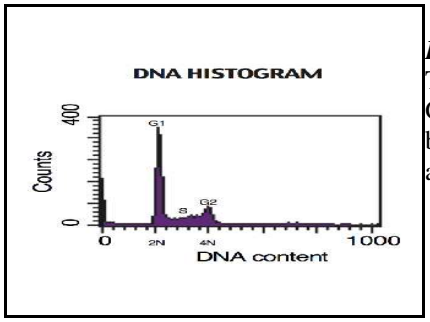

Figure 6. **A DNA histogram.**
The highest peak corresponds to the G1/G0 phase and the second peak to G2/M. In between the peaks is the S phase. Since cell doublets are excluded before this analysis, the cells in G2, which have 2 x the normal amount of DNA are not confused with cell doublets.

AIMS OF THIS STUDY

The aims of this study were to investigate the changes of global expression profiles and cell cycle progression due to *in vitro* hypoxia treatment of a breast cancer cell line. We wanted to investigate the time dependency of different responses to reveal which responses occur early due to the hypoxic treatment and which occur after a longer time.

MATERIALS AND METHODS

For a detailed description of the methods used see *Appendix B*.

CELL LINE

The breast carcinoma cell line MDA-MB-468 was obtained from ATCC (Rockville, MD) and derives from a pleural effusion from a 51-year-old black female patient with metastatic adenocarcinoma of the breast. MDA-MB-468 is estrogen receptor (ER) negative with a point mutation in codon 273 of the p53 gene resulting in an Arg -> His substitution.

CELL CULTURE

The cell line was cultured in RPMI 1640 medium, supplemented with 1mM NaPyr, 1.5 g/L NaBic, 10% FCS, 50 µg/ml streptomycin and 50 IU/ml penicillin.The cells were subcultured once a week and the medium was renewed twice between subculturing. The cultures were incubated at 37ºC in a water-saturated atmosphere containing 5% CO_2 in the air.

HYPOXIA TREATMENT

0.8 x 10^5 cells were seeded per cm^2 in 150-cm^2 and 25-cm^2 cell culture flasks the day before the treatment started such that they were logarithmically growing at the start of the experiment. At time point 0 the medium was renewed. The hypoxic cells were renewed with medium submitted to hypoxia for ~15 hrs and the control cells were renewed with regular medium. Hypoxic cells were placed in a humid hypoxic chamber containing 1% O_2, and 5% CO_2 at 37ºC and control cells were cultured with 20% O_2, 5% CO_2 at 37ºC. In the first experiment hypoxic and control cells were harvested after 0, 6 and 24 hrs by trypsinization. In the second experiment the cells were harvested after 0, 12 and 48 hrs. All harvested cells were washed twice in PBS and the cells used for RNA extraction were fixed in TRIzol, a mono-phasic solution of phenol and guanidine isothiocyanate (Life Technologies, Inc., Rockville, MD). The cells used for the flow cytometry experiment were fixed in 70% ethanol. All samples were stored at -20ºC until analysis.

FLOW CYTOMETRY

The cells were pelleted and resuspended in 800 µl Vindelov's propidium iodide solution (see *Appendix B*) for labeling the DNA, and incubated for 30 minutes at 4ºC. Immediately before the FCM analysis, the samples were suctioned through a cannula 3 times. The analysis was performed in a FACSCalibur flowcytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). A minimum of 10,000 cells per sample was collected and doublets were excluded by gating on FL2 width and FL2 area scatter. The fraction of G0/G1, S, G2-cells and dead cells were determined using manual gating based on the DNA content of the cells.

RNA ISOLATION, PURIFICATION AND LABELING

Total RNA was extracted from the cells previously fixed from the different time points and treatments. The RNA isolation and purification was performed by phenol-chloroform extraction and a subsequent purification using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturers' recommendations. The RNA was reversibly transcribed and coupled to Cy5 and Cy3 fluorochromes by indirect labeling using CyScribe Post-Labeling Kit from Amersham Pharmacia Biotech.

Cy5 was used to label the “reference cDNA” from time point 0 and Cy3 was used to label the “experimental cDNA” from the other time points. The labeled cDNA was subsequently purified by using the CyScribe GFX Purification Kit (Amersham Pharmacia Biotech).

MICROARRAY HYBRIDIZATION

MICROARRAY SLIDES

The arrays had previously been produced by using DNA targets amplified from Research Genetics Clones (ResGen, Invitrogen Corporation) by PCR. Amino-silane coated glass slides (GAPS II, Corning) printed with ~27 000 clones, by a MicroGridII robot (BioRobotics) equipped with MicroSpot2500 pins, were used for all hybridizations.

HYBRIDIZATION

The hybridization solutions were prepared by pooling the differentially labeled cDNAs and adding a blocking solution containing Cot-1 DNA, Poly dA and yeast tRNA. The samples were dried into pellets and resuspended in DIG-Easy/1% BSA.
8 hybridizations were performed;

hypoxia 6 hours vs reference (time point 0)
hypoxia 12 hours vs reference (time point 0)
hypoxia 24 hours vs reference (time point 0)
hypoxia 48 hours vs reference (time point 0)
normal 6 hours vs reference (time point 0)
normal 12 hours vs reference (time point 0)
normal 24 hours vs reference (time point 0)
normal 48 hours vs reference (time point 0)

The hybridization solution was added to a prehybridized, and rinsed microarray slide and incubated at 42ºC for 18 hours. After the hybridization the slides were washed with 2 x SSC and 0.1 % SDS, 1 x SSC, 0.1 x SSC and 0.06 x SSC and dried by centrifugation.

To reduce the background, the prehybridization was performed by using the The Pronto!™ Universal Pre-Soak treatment followed by Pre-Hybridization (Corning Lifesciences).

SCANNING THE SLIDES AND IMAGE ANALYSIS

The arrays were scanned with an Agilent DNA Microarray Scanner (Agilent Technologies, Palo Alto, CA) and image analysis and data extraction was performed using the GenePix Pro 3.0.6.86 software (Axon Instruments).

DATA ANALYSIS

The quantified data was loaded into the BioArray Software Environment (BASE) [35] where filtering and normalization were carried out.

QUALITY FILTER

To ensure that the spots of the array were true signals and not confused with noise a standard filtering of the data was performed as described:
Signal-to-Noise Ratio (SNR) median >= 3 for both channels (Cy3 and Cy5)
Spot diameter >= 60 pixels

where median SNR = $(\text{median}(I)_{spot} - \text{median}(I)_{background}) / \sigma(I)_{background}$ (I=intensity)
and Spot diameter = the amount of pixels containing signal for both channels (overlapping).

NORMALIZATION

The measured ratio for each spot is supposed to represent its relative expression level due to hypoxic or normal conditions, as compared to the reference. To balance the differences of the intensity ratios due to for example unequal quantities of starting RNA, differences in labeling or detection efficiencies between Cy3 and Cy5 or other systematic biases in the measured expression levels, a locally weighted linear regression (Lowess) normalization of the intensity ratios was performed [33].

PATTERN ANALYSIS

A selection filter was applied to ensure that each gene to be analyzed was of good quality in at least 7 of the 8 hybridizations (>=7). The transformed ratios (experiment/reference) were exported from BASE. Two different analyses were performed.

In Analysis 1, we wanted to distinguish the changes in gene expression caused by hypoxia treatment. Ratios of expression levels were calculated for all genes between the samples:

6H ratio / 6N ratio
12H ratio / 12N ratio
24H ratio / 24N ratio
48H ratio / 48N ratio

Each gene is then represented by four ratios (Hypoxia / Normal) corresponding to the different time points. This step ensures that a gene of interest is not interpreted as upregulated by hypoxia treatment if it also is upregulated to the same degree after the same amount of time during normal conditions.
In Analysis 2 we wanted to observe genes that fluctuate with time, not necessarily due to the hypoxia treatment. Here we used the ratios of the individual hybridizations directly,
hence each gene is represented by eight ratios (6H, 6N, 12H, 12N, 24H, 24N, 48H, 48N).

All ratios were transformed to logarithm base 2 in order to treat up- and downregulated genes in a symmetrical fashion, a gene upregulated by a factor 2 has a $\log_2$(ratio) of 1 and a gene downregulated by a factor 2 has a $\log_2$(ratio) of -1. A gene may be considered to be up/downregulated or unchanged by different criteria.Instead of using a certain fixed value as a determinant of the gene state we used the following model.

From the $\log_2$(ratio) of a gene, we assigned a value for that gene to be in a upregulated (+), downregulated (-) or unchanged (0) state, as follows.

$$P'_g(+) = exp\ [\ -(x_g - \tau*\sigma)^2 / \sigma^2\ ] \quad ;\quad x_g < \tau*\sigma$$
$$P'_g(+) = 1 \quad ;\quad x_g >= \tau*\sigma$$

$$P'_g(0) = exp\ [\ -x_g^2 / \sigma^2\ ]$$

$$P'_g(-) = exp\ [\ -(x_g + \tau*\sigma)^2 / \sigma^2\ ] \quad ;\quad x_g > -\tau*\sigma$$
$$P'_g(-) = 1 \quad ;\quad x_g <= -\tau*\sigma$$

where x_g is the $\log_2$(ratio) of the gene, g and $P'_g(+)$, $P'_g(-)$, $P'_g(0)$ are the values for g to be in an upregulated, unchanged or downregulated state.

The sum of these values were normalized to unity;

$$P'(+) + P'(-) + P'(0) = N \ \Rightarrow\ P(+) = P'(+) / N,\ P(-) = P'(-) / N,\ P(0) = P'(0) / N$$

In the following analyses we used $P(+)$, $P(-)$ and $P(0)$. These values depend on the parameters tau (τ) and sigma (σ). σ reflects the standard deviation value of the log 2(ratio) of all genes in an experiment, and is ideally set from a self-self hybridization where no genes are expected to be changed. Since we did not perform a self-self hybridization, we chose to use the standard deviation (σ) of N6 (cells growing for 6 hours in normal conditions) which was 0.4.
τ reflects the magnitude of a ratio required for a gene to be considered up or downregulated and we used $\tau = 3$. We used a threshold for P of 0.9 for a gene to be considered to be in a certain state with certainty. The false positive ratio for such a threshold and τ should also be estimated from self-self hybridizations. A missing value for a gene (expression in 7 of 8 samples) yields P(0) = 1 and P(+/-) = 0.

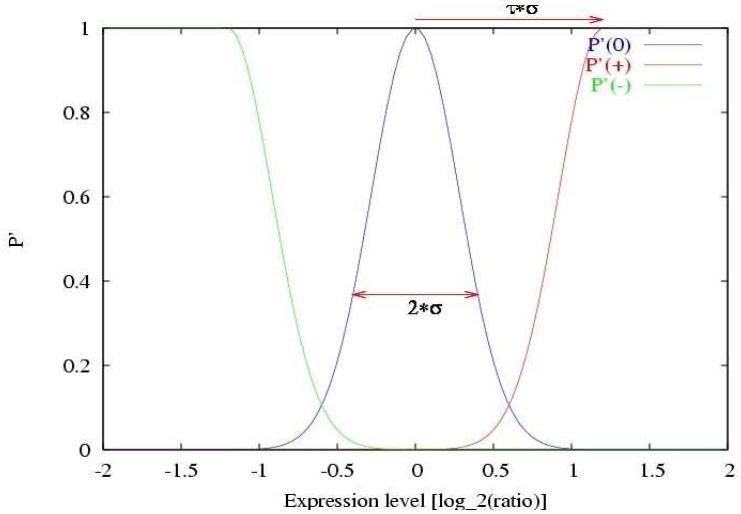

Figure 7. **A comprehensible view of the state values.**

To make this easier to interpret consider these examples of expression data:

If a gene has a log 2(ratio) of 1.3 it will have a high state value to be in an upregulated state (P'(+)).

If a gene has a log 2(ratio) of 0.1 it will have a high state value to be in an unchanged state (P'(0)).

In Analysis 1 we were interested in genes which are upregulated and downregulated in certain fashions;

6	12	24	48 (hours of treatment)
+	+	+	+ = upregulated at all time points by hypoxia.
0	+	+	+ = upregulated after 12 hrs by hypoxia.
0	0	+	+ = upregulated after 24 hrs by hypoxia.

For downregulated genes we were interested in the same patterns as above (downregulated in all time points, downregulated after 12 hrs, etc).
In Analysis 2 we were interested in genes that fluctuate in a similar manner irrespective of the treatment, but dependent on the time-length of treatment (normal or hypoxia).

6H	12H	24H	48H	6N	12N	24N	48N (6N= 6 hours normal, 6H= 6hours hypoxia etc).
+	-	+	-	+	-	+	- = 6 hrs up, 12 hrs down, 24 hrs up, 48 hrs down
+	-	+	0	+	-	+	- = 6 hrs up, 12 hrs down, 24 hrs up, 48 hrs down in normal, unchanged in hypoxia.

We assigned a pattern value for each gene to exhibit the patterns described above by using the product of the state values (*P*) assigned for each experiment. For the patterns in Analysis 1 we used a threshold of 0.9^4 as a filter to distinguish genes which exhibit the pattern of interest with certainty. For the patterns of Analysis 2 the threshold was set to 0.9^8.

RESULTS AND DISCUSSION

The results from the cell cycle analysis are depicted in *Figures 8-9 (Appendix A)* and further discussed below.The results from the pattern analyses are depicted in *Figures 10-15 (Appendix A).*The genes highlighted in bold in the figures are further discussed below.

The results from the microarray time-course experiment show that it is of great importance to consider the time-length of hypoxia treatment when interpreting the result of any such experiment. In the following section genes which are described as *e.g.* upregulated are upregulated in the samples submitted to hypoxic treatment compared to the sample grown under normal conditions. Some general observations from our microarray experiment are discussed here:

- Fewer genes are induced or reduced after 6 hours compared to after 12 or 24 hours.
These observations allow us to distinguish between pathways which are transcriptionally induced early or late after hypoxic treatment. For example, certain genes involved in glucose metabolism are upregulated after 6 hours and genes involved in cell cycle progression are downregulated or upregulated after 24 hours, as described below.

- Overall, more genes are upregulated than downregulated in our data and practically all downregulated genes are reduced at or later than 24 hours of treatment.

- There are "cyclic genes" which are regulated by the time length from time point 0 rather than hypoxia treatment.

Some representatives of the genes which are regulated by hypoxia treatment in our experiment are discussed below, divided into their respective "functional" pathways. Together with the data from the flow cytometry experiments some conclusions can be drawn;

Cell cycle

In our time-course microarray data we observed a downregulation after 24 hours (00--, *Figure 13*) of several genes involved in cell cycle progression;

Cyclin E1 (G1-> S phase)
Cyclin D1 (G0 -> S phase)
Cdc25B (activates CDKs)
M-phase phosphoprotein 6 (G2 -> M-phase)
RAN (S phase and M phase)

We also saw an upregulation after 24 hours (00++, *Figure 12*) of Growth arrest and DNA-damage-inducible, beta (GADD-beta), which is known to be upregulated by stress and to induce cell cycle arrest to allow for DNA repair [36]. Our results from the flow cytometry do not imply an arrest in any stage of the cell cycle (*Figure 9*), which may be due to the fact that the MDA-468 cell-line has a non-functional Rb and exhibits a disturbed regulation of the cell cycle. The results from the microarray data may however suggest that the cell cycle is slowed down in the cells submitted to hypoxia. The PI flow cytometry experiments only provide a fixed image of the cell cycle phase distribution and measurement of DNA content alone gives the proportion of cells in the S phase, but does not reveal their capacity to replicate DNA or their progression rate through the S phase. To investigate the hypothesis described above, bivariate DNA/BrdUrd labeling can be used to analyze the lengths of the G1, S and G2 phases and also the rate of the G1/S transition [37] yielding a kinetic image of the cell cycle progression.

Apoptosis

The results from the flow cytometry indicate that there is a tendency towards an increase in the number of dead cells after hypoxia treatment for all time points investigated *(Figure 9*). Yet, this assay does not distinguish between apoptotic and necrotic cells.
The time-course microarray data showed an upregulation at all time points (++++, *Figure 10*) for BNIP3 which is concidered to be a pro-apoptotic protein, as previously mentioned. However, after 24 hours we observed an induction (00++, *Figure 12*) of several anti-apoptotic genes, *e.g.* Apoptosis Inhibitor 2 (API2), TNF-alpha induced protein 3 (inhibits TNF-mediated cell death) and EGFR. Hence, we observe a possible upregulation of both pro- & anti-apoptotic genes.

Due to these results one may suspect that cells suffer an early increase in apoptosis during hypoxia which is later on reduced by the induction of apoptotic inhibitors. However, the microarray experiments only show the induction or reduction of mRNA levels and do not reflect the protein activity. To further investigate whether there is an increase or reductions in apoptosis due to hypoxia additional experiments need to be performed which distinguish necrosis from apoptosis.

Glucose metabolism

The microarray data suggest an upregulation of genes involved in glucose transport through the plasma membrane, as well as an upregulation of almost all enzymes involved in the glycolysis;

After 6 hours we observed a consistent upregulation of Glucose Transporter 1 (GLUT-1), Hexokinase 2, and Enolase 2 (++++, *Figure 10*). After 12 hours of hypoxia, Phosphofruktokinase (PPK), Phosphoglycerate kinase (PGK) and Lactate dehydrogenase were upregulated (0+++, *Figure 11*). After 24 hours, Aldolase A and B, Enolase 3 and Glyceraldehyde 3-phosphate dehydrogenase (G3PD) were upregulated (00++, *Figure 12*).

As a conclusion, enzymes involved in the glycolysis and glucose transport were markedly upregulated due to hypoxia treatment. The time point of induction for the genes differed, although several are known to be induced by HIF-1 (see Box 1). This may be due to regulation by other genes as well.

HIF-1 and HIF-2

As mentioned previously, HIF-1α is considered to be mainly regulated at the protein level. In our experiment, we observed an upregulation after 24 hours of HIF-2, a close relative to HIF-1, suggesting a different mechanism for transcriptional regulation of these two homologues.

HIF-1 responsive genes

We did not perform any experimental assay to verify the level of HIF-1 protein. However, we observed the induction of several HIF-1 responsive genes (see Box 1), which suggest that we should have increasing levels of HIF-1 protein in the cells submitted to hypoxia. To mention a few HIF-1 responsive genes, after 6 hours we observed an increase in BNIP3, GLUT1, Enolase 2, proline 4-hydroxylase, IGFB3, Hexokinase 2, Carbonic Anhydrase IX and Endothelin 2 (++++, *Figure 10*).

Angiogenesis

Interestingly, the microarray data shows an upregulation due to hypoxia of several genes involved in angiogenesis. After 6 hours of hypoxia, tissue factor (TF) was upregulated (++++, *Figure 10*) and after 24 hours, epidermal growth factor receptor (EGFR) and Ephrin B2 receptor (Eph2B) were upregulated (00++, *Figure 12*). These genes have all been shown to induce angiogenesis by different mechanisms [38].

Other interesting genes

We saw an upregulation after 6 hours (++++, *Figure 10*) of Lysyl Oxidase-like 2 (LOXL2). LOXL2 initiates the covalent cross-linking of collagens and elastin in extracellular matrices. In a study by Kirschmann *et al.,* [39] it was demonstrated that LOXL2 mRNA expression only was observed in breast cancer cells with a highly invasive/metastatic phenotype and not in poorly invasive/nonmetastatic breast cancer cells. Additionally, the study demonstrated that Lysyl Oxidase (LOX) was directly involved in breast cancer invasion. We see an increase in LOX mRNA after 24 hours (00++, *Figure 12*). To my knowledge, LOX and LOXL2 have not previously been suggested to be induced by hypoxia.

"Cyclic genes"

The patterns of genes which were observed to be upregulated and downregulated in cycles

(+ - + - + - + -, *Figure 14*) (+ - + 0 + - + -, *Figure 15*) were surprising. Most genes exhibiting these patterns of expression were also up/downreulated to a very high degree which is hard to explain. Apparently, most of the genes exhibiting these patterns of expression are either chemokines or induced by TNF, or both. For example, GRO1 and GRO3 oncogene, MCP-1, MIP-3 alpha and IL-8 are chemokines and Serum amyloid A2 (SAA2) and antitrypsin are regulated by TNF. TNF itself also showed this pattern of expression. Some of these genes have been described to be induced by hypoxia and to play a role in angiogenesis [40]. This pattern of expression needs to be verified to draw any further conclusions.

CONCLUSIONS AND FUTURE PROSPECTS

The purpose of this project was to investigate the *in vitro* effect of hypoxia on a breast cancer cell line by performing a time course experiment and investigating the changes in the global expression profiles using cDNA microarrays and the changes in cell cycle distribution by flow cytometry. Hypoxia treatment affected the expression of genes involved in glucose metabolism, apoptosis, cell cycle progression and angiogenesis. The time series investigation revealed that genes involved in glycolysis are induced earlier at a transciptional level compared to genes involved in cell cycle progression. Downregulation of genes due to hypoxia occurred almost exclusively after 24 hours. These experiments need to be replicated and validated for further interpretations.

Since cDNA microarrays only measure changes in mRNA expression it is of interest to investigate the changes in protein levels of interesting genes (for example lysyl oxidase-like 2) by for example immunohistochemistry to see if the protein expression coincides with HIF-1 protein expression.

To further investigate the effect of hypoxia on the cell cycle progression it will also be interesting to investigate the cell cycle kinetic properties by means of bivariate DNA/BrdUrd staining (as described in *Discussion*).

In addition, it is of great interest to further develop the analysis method of time course microarray data, for example by including more possible states of gene expression maybe by including strongly upregulated (+ +) and downregulated (- -) states. which would generate more possible patterns of expression.

Hypoxia has been indicated to reduce the expression of the estrogen receptor [11] which may be a part of the explanation to the lack of response to hormone therapy in a subset of ER positive breast cancers. Therefore it will be of interest to perform similar experiments of another breast cancer cell line which contains a functional estrogen receptor. The response to hypoxia may also differ in breast cancer cell lines which contain a *BRCA1* mutation since the BRCA1 protein is implicated in different DNA repair mechanisms [3].

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APPENDIX A

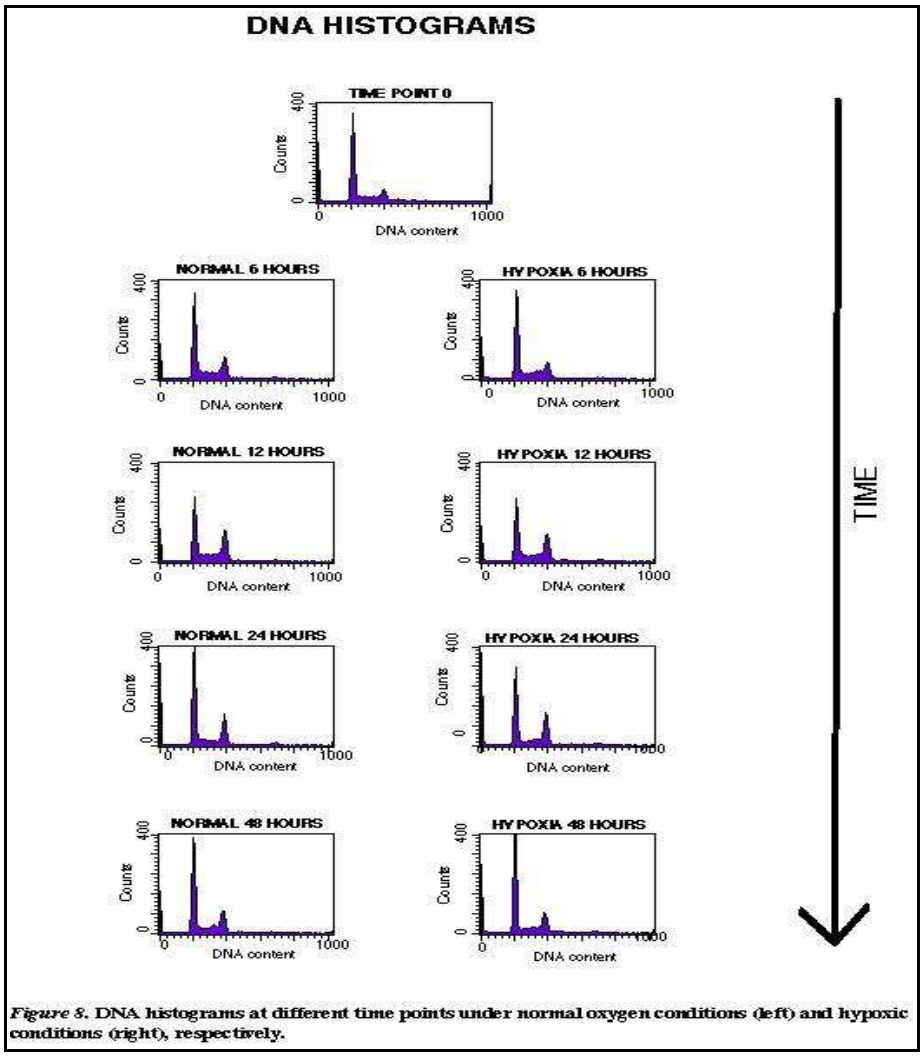


Figure 8. **DNA histograms at different time points under normal oxygen conditions (left) and hypoxic conditions (right), respectively.**

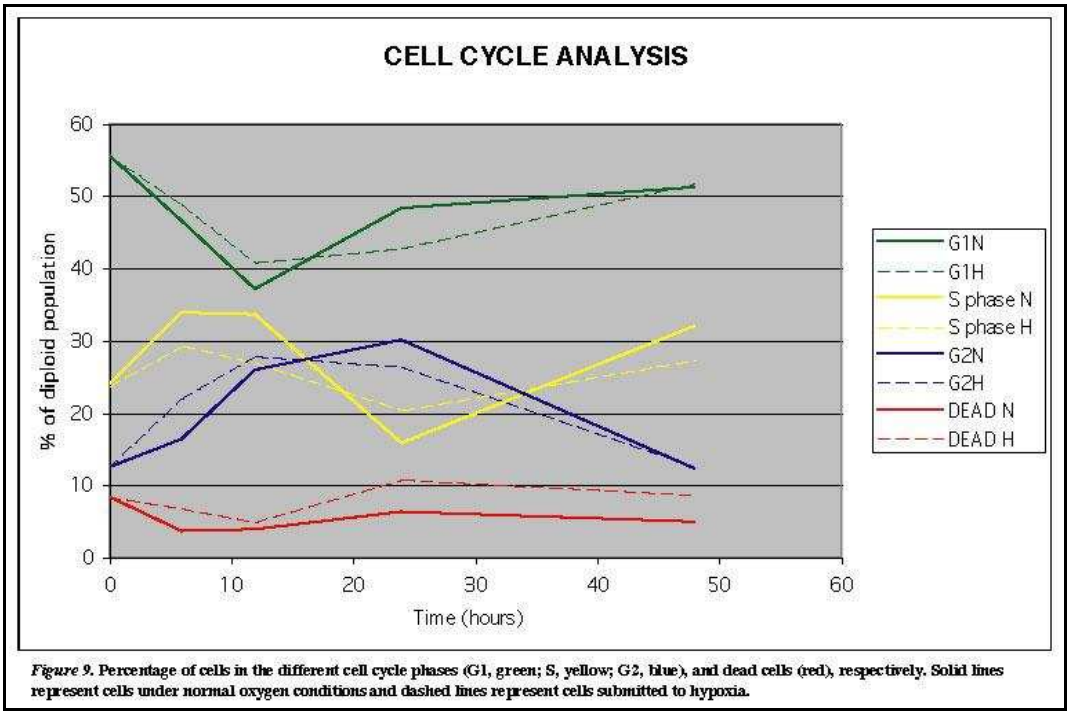
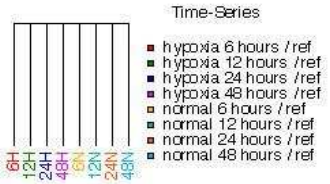
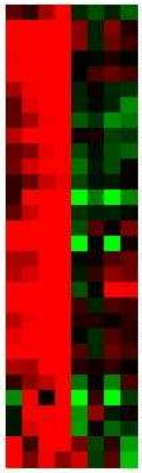


Figure 9. **Percentage of cells in the different cell cycle phases (G1, green; S, yellow; G2, blue), and dead cells (red), respectively. Solid lines represent cells under normal oxygen conditions and dashed lines represent cells submitted to hypoxia.**

GENES UPREGULATED AFTER 6 HOURS, ++++

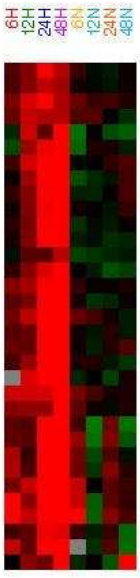




ID	Name
308497	Hypothetical protein HT036
842863	**N-myc downstream regulated**
1637282	**Hexokinase 2**
377452	Multiple cluster assignments (2)
898218	**Insulin-like growth factor binding protein 3**
2547341	**Solute carrier family 2 (facilitated glucose ...**
882506	**Lysyl oxidase-like 2**
207358	**Solute carrier family 2 (facilitated glucose ...**
453589	**Solute carrier family 2 (facilitated glucose ...**
233581	Huntingtin interacting protein 2
359982	**BCL2/adenovirus E1B 19kD-interacting protein 3**
744983	Papillomavirus regulatory factor PRF-1
1643566	**Carbonic anhydrase IX**
2308429	Protein tyrosine phosphatase, receptor type, f ...
361190	Pyruvate dehydrogenase kinase, isoenzyme 1
1693357	**Endothelin 2**
377419	Phosphoinositide-3-kinase, regulatory subunit, ...
469297	Basic helix-loop-helix domain containing, class B, 2
813645	Hypothetical protein
838802	**Procollagen-proline, 2-oxoglutarate 4-dioxygenase ...**
491311	EGL nine (C.elegans) homolog 3
135900	KIAA1376 protein
134976	Hypothetical protein from EUROIMAGE 1669387
490995	**Procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysin**
196435	Weakly similar to KIAA0738 protein [H.sapiens]
773138	Hypothetical protein PRO1489
770910	E74-like factor 3 (ets domain transcription factor, ...
789147	**Enolase 2, (gamma, neuronal)**
2252954	Enhancer of filamentation 1 (cas-like docking; ...
1928791	**Coagulation factor III (thromboplastin, tissue factor)**

Figure 10. **Genes upregulated by hypoxia at all time points.**

GENES UPREGULATED AFTER 12 HOURS, 0+++



ID	Name
324891	Degenerative spermatocyte (homolog Drosophila; lipid ...
795207	Ku70-binding protein 3
824983	Adenylate kinase 3
755416	Multiple cluster assignments (2)
503602	Hypothetical protein PRO1489
2110547	ESTs
435219	MAX-interacting protein 1
277611	MAX-interacting protein 1
471655	Multiple cluster assignments (2)
277480	Homo sapiens cDNA FLJ25045 fis, clone CBL03591
136303	RAR (RAS like GTPASE)
949939	**Phosphoglycerate kinase 1**
178153	Multiple cluster assignments (2)
950682	**Phosphofructokinase, platelet**
489393	Hypothetical protein 628
768944	**Phosphoglycerate kinase 1**
897567	**Lactate dehydrogenase A**
50882	Endothelin converting enzyme 1
845658	**Hypoxia-inducible protein 2**
795178	**Lactate dehydrogenase C**
1493252	Hypothetical protein FLJ11200
41345	Rearranged L-myc fusion sequence
22920	No cluster assignment
241705	Phosphorylase, glycogen; liver (Hers disease, glycogen ...
431280	No cluster assignment
347546	ERO1-like (S. cerevisiae)
276816	KIAA1718 protein
730072	Hypothetical protein 628
1572614	Interferon gamma receptor 2 (interferon gamma transduc
785575	Interferon gamma receptor 2 (interferon gamma transduc
810331	Multiple cluster assignments (2)
324785	Procollagen-proline, 2-oxoglutarate 4-dioxygenase ...
502198	Multiple cluster assignments (2)

Figure 11. **Genes upregulated by hypoxia after 12 hours.**

GENES INDUCED AFTER 24 HOURS, 00++ (I)

ID	Name
1031919	Weakly similar to AF126780 1 retinal short-chain ...
814240	Forkhead box O3A
280934	Mevalonate (diphospho) decarboxylase
2325916	Calcium/calmodulin-dependent serine protein kinase ...
4900502	
1662163	EST
324154	Selenoprotein N, 1
274677	ESTs
2545711	**Enolase 2, (gamma, neuronal)**
788232	Sestrin 2
277186	Phosphatidylinositol transfer protein, membrane-associat
140044	Transducin-like enhancer of split 4, homolog of ...
2030270	Novel human gene mapping to chomosome 1
1868626	**Phosphofructokinase, liver**
1499828	Fucosyltransferase 1 (galactoside ...
1637756	**Enolase 1, (alpha)**
1613496	No cluster assignment
814417	Hypothetical protein HDCMC04P
1564300	Histone deacetylase 3
489047	Hypothetical protein MGC15437
470216	Multiple cluster assignments (2)
744388	KIAA1041 protein
241197	**Epidermal growth factor receptor (avian erythroblastic ...**
324861	**Epidermal growth factor receptor (avian erythroblastic ...**
485195	Claudin 3
562778	LIM protein (similar to rat protein kinase C-binding enigm
271198	Nuclear receptor subfamily 3, group C, member 1
46930	ESTs
825092	Cystathionase (cystathionine gamma-lyase)
2565691	HIV TAT specific factor 1
22908	Weakly similar to I38022 hypothetical protein [H.sapiens]
815124	Carcinoembryonic antigen-related cell adhesion molecule
356631	KIAA1361 protein
155838	Membrane interacting protein of RGS16
246661	DKFZP564A122 protein
2029453	DKFZP564A122 protein
366389	DKFZP564A122 protein
435970	ESTs
82297	Hypothetical protein FLJ14642
1525305	KIAA0692 protein
826254	Ubiquitin-conjugating enzyme E2H (homologous to yeast

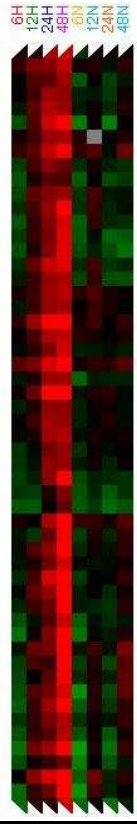
GENES UPREGULATED AFTER 24 HOURS, 00++ (II)

769947	ESTs
2435216	Phosphorylase, glycogen; liver (Hers disease, glycogen .
825295	Low density lipoprotein receptor (familial ...
811079	Chromosome 1 open reading frame 24
1605426	Hypothetical protein FLJ13352
257011	Multiple cluster assignments (2)
773278	DnaJ (Hsp40) homolog, subfamily B, member 9
135716	ESTs
282108	A kinase (PRKA) anchor protein 2
2569196	Microtubule-associated protein 4
788667	Multiple cluster assignments (2)
1474023	Jagged 1 (Alagille syndrome)
773301	Cadherin 3, type 1, P-cadherin (placental)
289016	Homo sapiens cDNA FLJ31349 fis, clone MESAN200009
149539	MAPK phosphatase-7
2074634	ESTs
139861	CAMP responsive element binding protein-like 2
2449343	Protein tyrosine phosphatase, receptor type, H
32522	Weakly similar to I38022 hypothetical protein [H.sapiens]
845432	Rag D protein
2159983	Pro-oncosis receptor inducing membrane injury gene
131452	KIAA1102 protein
138693	No cluster assignment
364992	KIAA0914 gene product
46411	Homo sapiens cDNA: FLJ22030 fis, clone HEP08669
262695	A kinase (PRKA) anchor protein 2
594630	Short-chain dehydrogenase/reductase 1
361048	Multiple cluster assignments (2)
346009	**Phosphofructokinase, liver**
1626279	Chromosome 22 open reading frame 4
878111	clone MGC:17491 IMAGE:3452986, mRNA, complete cd
2545705	Calponin 3, acidic
366887	Bone morphogenetic protein 7 (osteogenic protein 1)
681917	Interleukin 1 receptor accessory protein
2550001	Protein phosphatase 2 (formerly 2A), regulatory subunit .
448191	Chromosome 1 open reading frame 24
310356	Angiopoietin-like 4
490612	RAN binding protein 7
2165360	Pvt-1 (murine) oncogene homolog, MYC activator
450079	Homo sapiens cDNA: FLJ23593 fis, clone LNG14821
343932	Hypothetical protein FLJ21939 similar to 5-azacytidine ...
1474684	Ephrin-A1
814135	ESTs
430213	ESTs
855745	Highly similar to G2HU Ig gamma-2 chain C region [H.sa
770394	Fc fragment of IgG, receptor, transporter, alpha
141815	Jagged 1 (Alagille syndrome)
859574	Phosphatidylinositol transfer protein, membrane-associat
27801	Stress 70 protein chaperone, microsome-associated, 60k
234183	ESTs
813751	Sialyltransferase 4C (beta-galactosidase ...
843291	Target of myb1 (chicken) homolog
71087	V-maf musculoaponeurotic fibrosarcoma (avian) oncogen
240295	Hypothetical protein FLJ23399

GENES UPREGULATED AFTER 24 HOURS, 00++ (III)

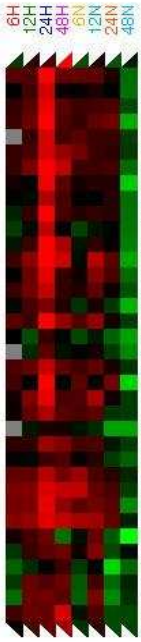
2110750	KIAA0874 protein
377363	Homo sapiens cDNA FLJ11639 fis, clone HEMBA100432
208699	Multiple cluster assignments (2)
125191	ESTs
488434	Likely ortholog of mouse coiled coil forming protein 1
1475115	No cluster assignment
345935	Multiple cluster assignments (2)
138234	ESTs
191826	Mitochondrial solute carrier
194161	Actinin, alpha 4
782768	Weakly similar to I38022 hypothetical protein [H.sapiens]
345849	**Lysyl oxidase**
428192	ESTs
730555	DnaJ (Hsp40) homolog, subfamily B, member 1
133454	Amino acid transporter 2
345103	EphB2
132144	Zinc finger protein 354A
506165	Cdc42 effector protein 3
292612	Multiple cluster assignments (2)
713078	Hypothetical protein MGC4618
795877	Serum-inducible kinase
1292581	No cluster assignment
297392	Multiple cluster assignments (2)
122178	Highly similar to S68178 mixed-lineage protein kinase 2 .
842836	Serine (or cysteine) proteinase inhibitor, clade B ...
471219	No cluster assignment
431535	ESTs
290555	ESTs
746234	Moderately similar to I38022 hypothetical protein ...
161272	No cluster assignment
841620	Dihydropyrimidinase-like 2
4102878	
247117	Alanine-glyoxylate aminotransferase (oxalosis I; ...
129585	Moderately similar to Cd-7 Metallothionein-2 [H.sapiens]
202535	Metallothionein 1G
1899230	H.sapiens mRNA for metallothionein isoform 1R
202706	Homo sapiens mRNA; cDNA DKFZp434N2116 (from clo
279824	Homo sapiens cDNA FLJ13069 fis, clone NT2RP3001 75
279460	Epsilon-tubulin
80484	P8 protein (candidate of metastasis 1)
856878	ESTs
280740	C-myc promoter-binding protein
1623210	Homo sapiens cDNA: FLJ21771 fis, clone COLF7779
491418	Multiple cluster assignments (2)
130892	EH-domain containing 3
241874	Hypothetical protein FLJ12592
137211	Chromodomain helicase DNA binding protein 2
815297	Multiple cluster assignments (2)
1592837	Interferon induced transmembrane protein 2 (1-8D)
366071	BBP-like protein 1
1606321	DKFZP586J0619 protein

GENES UPREGULATED AFTER 24 HOURS, 00++ (IV)



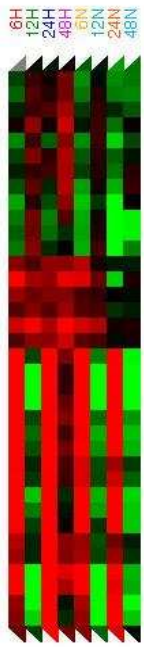
128833	Protein kinase, lysine deficient 1
609111	DKFZP434F195 protein
810263	Homo sapiens cDNA: FLJ23241 fis, clone COL01375
415066	KIAA0355 gene product
309493	SH3 protein interacting with Nck, 90 kDa
840818	Filamin B, beta (actin-binding protein-278)
898208	DKFZP727M231 protein
2222997	Human pancreatic mucin mRNA, complete cds
756533	Basigin (OK blood group)
2440877	Bone morphogenetic protein 7 (osteogenic protein 1)
1460141	X 001 protein
241139	Human c-abl gene, complete cds
2273381	Human DNA sequence from clone 108K11 on chromoso
741474	**Glucose phosphate isomerase**
1469803	**glucose phosphate isomerase**
449112	Highly similar to 1203217A deh...
449126	ESTs
1704527	Breakpoint cluster region protein, uterine leiomyoma, 2
1874052	Olfactory receptor, family 2, subfamily A, member 7
4301677	
140018	LBP protein 32
2471503	SH3-domain binding protein 2
283430	KIAA1320 protein
254310	ESTs
878578	**Aldolase A, fructose-bisphosphate**
2018418	EXPRESSION INFORMATION
1590626	**Aldolase B, fructose-bisphosphate**
35077	Nuclear protein, marker for differentiated aortic smooth ..
35185	Gamma-aminobutyric acid (GABA) A receptor, beta 1
34849	Eukaryotic translation elongation factor 2
24339	Multiple cluster assignments (2)
486086	SH3-domain kinase binding protein 1
1604005	SH3-domain kinase binding protein 1
110430	ESTs
449053	Hypothetical protein FLJ12270
825207	ESTs
136560	KIAA0453 protein
2413337	Sortilin-related receptor, L(DLR class) A repeats-containi
858153	Nuclear factor, interleukin 3 regulated
449275	No cluster assignment
427767	Homo sapiens clone IMAGE:119716, mRNA sequence
2461050	Lectin, galactoside-binding, soluble, 1 (galectin 1)
745136	ESTs
745090	Hypothetical protein FLJ23033
283919	H2A histone family, member L
2297394	Hypothetical protein FLJ13612
140806	Peptidylglycine alpha-amidating monooxygenase
347331	Homo sapiens cDNA: FLJ21447 fis, clone COL04468
205303	Sorcin
172785	NAG-5 protein
730439	Weakly similar to MCAT_HUMAN MITOCHONDRIAL ...
809494	CD151 antigen
1731860	**Growth arrest and DNA-damage-inducible, beta**
2108359	Arginyl aminopeptidase (aminopeptidase B)

GENES UPREGULATED AFTER 24 HOURS, 00++ (V)



1606557	Four and a half LIM domains 2
2073188	Moderately similar to KPCM_HUMAN PROTEIN KINASE
25988	Eukaryotic translation initiation factor 4 gamma, 1
1640707	SWI/SNF related, matrix associated, actin dependent ...
1628456	Nasopharyngeal carcinoma susceptibility protein
2248780	KIAA0731 protein
127682	Rec
2512263	Enolase 3, (beta, muscle)
2052032	Myosin X
812967	Tetraspan 5
1910785	ATPase, Class VI, type 11A
1500416	ATPase, Class VI, type 11A
124661	No cluster assignment
1486028	Weakly similar to 2108276A ssDNA-binding protein ...
773479	FK506-binding protein 1A (12kD)
33386	Human mRNA for KIAA0120 gene, complete cds
854079	Actinin, alpha 1
1910465	Hypothetical protein
814893	Homo sapiens mRNA; cDNA DKFZp564H0764 (from clo
366105	Protein phosphatase 1, regulatory subunit 10
812266	Mannosidase, alpha, class 1A, member 2
2125838	HLA-G histocompatibility antigen, class I, G
240249	Amyloid beta (A4) precursor-like protein 2
202799	Multiple cluster assignments (2)
502664	Ras-induced senescence 1
289551	Fragile X mental retardation, autosomal homolog 1
263892	Eukaryotic translation initiation factor 5A
2305636	Cadherin 1, type 1, E-cadherin (epithelial)
292731	HBV pX associated protein-8
814976	KIAA0414 protein
814303	Kinectin 1 (kinesin receptor)
133717	Thyroid hormone receptor-associated protein, 150 kDa s
2463082	Nuclear receptor interacting protein 1
109316	Serine (or cysteine) proteinase inhibitor, clade A ...
435371	No cluster assignment
897781	Keratin 8
79216	AHNAK nucleoprotein (desmoyokin)
2577077	Aminopeptidase puromycin sensitive

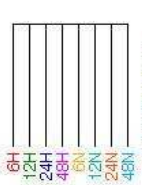
GENES UPREGULATED AFTER 24 HOURS, 00++ (VI)

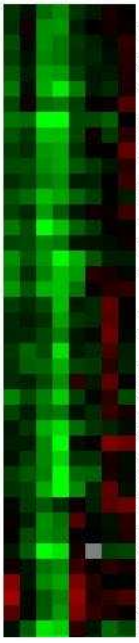


842846	Tissue inhibitor of metalloproteinase 2
1457276	Homo sapiens cDNA: FLJ22182 fis, clone HRC00953
1472797	Hypothetical protein FLJ10718
782501	Hypothetical protein PP1665
234011	Plakophilin 2
810813	S100 calcium-binding protein A2
361570	Popeye protein 3
855610	Synaptopodin
342008	Keratin 13
782545	Hypothetical protein EDAG-1
366889	H.sapiens gene for cytokeratin 17
1048993	No cluster assignment
183602	Keratin 14 (epidermolysis bullosa simplex, ...
299059	PRO1914 protein
814798	Aldehyde dehydrogenase 1 family, member A3
33967	Hypothetical protein FLJ11220
1467207	Sec23-interacting protein p125
24958	Homo sapiens mRNA; cDNA DKFZp434C2016 (from clo
34010	Weakly similar to ALU1_HUMAN ALU SUBFAMILY J SE
2458975	Inhibin, beta A (activin A, activin AB alpha polypeptide)
141806	Tumor necrosis factor, alpha-induced protein 3
2508563	Tumor necrosis factor, alpha-induced protein 3
770670	Tumor necrosis factor, alpha-induced protein 3
145112	Intercellular adhesion molecule 1 (CD54), human ...
293413	Intercellular adhesion molecule 1 (CD54), human ...
2246281	Peptidyl arginine deiminase, type V
2243734	ESTs
2388761	Human lyn mRNA encoding a tyrosine kinase
201890	Baculoviral IAP repeat-containing 3
127032	Baculoviral IAP repeat-containing 3
754355	Sema domain, seven thrombospondin repeats (type 1 an
491694	Hypothetical protein FLJ23399
133988	Fatty-acid-Coenzyme A ligase, long-chain 4
2543464	Serine (or cysteine) proteinase inhibitor, clade A ...
450533	Serine (or cysteine) proteinase inhibitor, clade A ...
66437	Lipopolysaccharide-binding protein
84730	Lipopolysaccharide-binding protein
51814	Cystatin B (stefin B)

Figure 12. **Genes upregulated by hypoxia after 24 hours.**

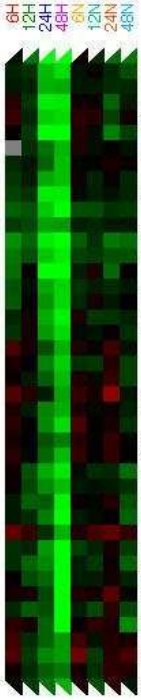
GENES DOWNREGULATED AFTER 24 HOURS, 00-- (I)





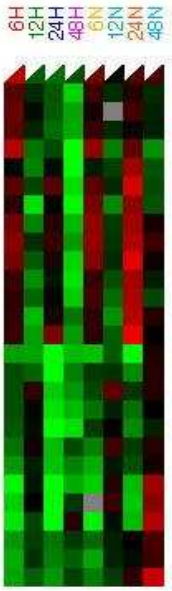
ID	Name
188422	Hypothetical protein FLJ20085
206118	ESTs
1558655	Hypothetical protein FLJ22316
2018517	Weakly similar to I57588 HSrel-1 [H.sapiens]
795612	Serologically defined colon cancer antigen 33
825641	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, ...
1636907	Transcription termination factor-like protein
283173	Homo sapiens PAC clone RP1-130H16 from 22q12.1-qte
824927	Similar to RIKEN cDNA 2510006C20 gene, clone MGC:2
745283	Weakly similar to Y961_HUMAN HYPOTHETICAL ZINC
795809	Ubiquitin-conjugating enzyme E2E 3 (homologous to yea
416374	Tetraspan NET-6 protein
814258	Nuclear transcription factor Y, gamma
1500162	ESTs
770388	Claudin 4
204790	Hypothetical protein
143426	Ras homolog gene family, member B
395539	EST
1032405	Bone morphogenetic protein 5
1521712	ESTs
759184	Similar to RIKEN cDNA 2610030J16 gene, clone MGC:2
51420	ESTs
1492287	Homo sapiens cDNA FLJ11658 fis, clone HEMBA100457
283995	Homo sapiens mRNA; cDNA DKFZp761I1912 (from clon
812197	KIAA1383 protein
809720	Centromere protein B (80kD)
448325	Hypothetical protein MGC4604
283292	Hypothetical protein FLJ10535
755299	Immediate early protein
813815	Hypothetical protein FLJ12770
2017860	Homo sapiens cDNA FLJ31007 fis, clone HLUNG200012
757873	**Cyclin-dependent kinase 5, regulatory subunit 1 (p35)**
824260	ESTs
1642186	Diptheria toxin resistance protein required for ...
827141	Weakly similar to T23976 hypothetical protein R06F6.5a
823771	CDC2-related protein kinase 7
243878	Endoplasmic reticulum resident protein 58; hypothetical .
77539	Small inducible cytokine subfamily A (Cys-Cys), member
841641	**Cyclin D1 (PRAD1: parathyroid adenomatosis 1)**
882510	Karyopherin alpha 2 (RAG cohort 1, importin alpha 1)
825695	Hypothetical protein

GENES DOWNREGULATED AFTER 24 HOURS, 00-- (II)



160233	Thymopoietin
810156	Deoxythymidylate kinase (thymidylate kinase)
2506585	Proteasome (prosome, macropain) activator subunit 3 ...
429647	LanC (bacterial lantibiotic synthetase component C)-like
811956	**RAN, member RAS oncogene family**
796646	Ornithine decarboxylase 1
288667	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 28
767171	Hypothetical protein
280970	Nucleolar protein 1 (120kD)
594759	Similar to RIKEN cDNA 1110001A12 gene, clone MGC:2
1321677	No cluster assignment
435415	Mitochondrial ribosomal protein L50
856796	Spermidine synthase
758360	ESTs
786686	ESTs
448371	CGI-01 protein
298937	Hypothetical protein dJ551D2.5
68950	**Cyclin E1**
342479	Myotubularin related protein 8
1239953	No cluster assignment
1949085	Carbonyl reductase 3
1541734	ESTs
305697	Homo sapiens cDNA FLJ32089 fis, clone OCBBF200071
810316	Lipidosin
73702	No cluster assignment
263846	Protein phosphatase 2 (formerly 2A), regulatory subunit .
878640	Moderately similar to S02826 nonhistone chromosomal .
431214	Hypothetical protein FLJ12484
26443	GTT1 protein
786067	**Cell division cycle 25B**
236263	No cluster assignment
1467244	Similar to neuregulin 4, clone MGC:22713 IMAGE:40716
358162	Protein predicted by clone 23627
2015354	Neuro-oncological ventral antigen 1
362985	ESTs
256664	H2A histone family, member X
213176	Sterol O-acyltransferase 2
624627	Ribonucleotide reductase M2 polypeptide
2420316	**M-phase phosphoprotein 6**
278430	Kinesin family member 5C
273625	Hypothetical protein
23461	Homo sapiens mRNA full length insert cDNA clone ...

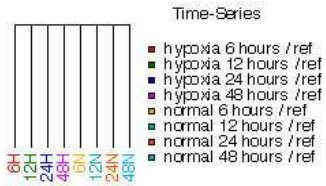
GENES DOWNREGULATED AFTER 24 HOURS, 00-- (III)

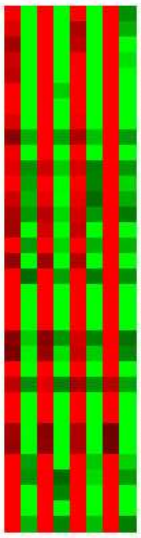


815542	Myxovirus (influenza) resistance 1, homolog of murine ...
2418897	Phorbolin (similar to apolipoprotein B mRNA editing prote
209279	Hypothetical protein DKFZp586I0418
1048696	Phorbolin-like protein MDS019
712292	Multiple cluster assignments (2)
960615	No cluster assignment
433310	EST
2445159	Sema domain, immunoglobulin domain (Ig), short basic ...
252515	Kynureninase (L-kynurenine hydrolase)
435890	ESTs
2298080	Kynureninase (L-kynurenine hydrolase)
359701	Homo sapiens beta-1 adrenergic receptor mRNA, 3' UTR
391984	Homo sapiens beta-1 adrenergic receptor mRNA, 3' UTR
1456937	Oviductal glycoprotein 1, 120kD (mucin 9, oviductin)
295843	Cytochrome P450, subfamily XXVIIA (steroid ...
1468263	ADP-ribosyltransferase 3
23221	Homo sapiens cDNA FLJ30162 fis, clone BRACE200056
193892	Paternally expressed 10
211417	Solute carrier family 25 (mitochondrial carrier; ...
855243	Ribonuclease P, 40kD subunit
210575	Visinin-like 1
840567	Transmembrane 4 superfamily member 1
812294	ESTs
51011	Guanylate cyclase 1, soluble, alpha 3
839736	Crystallin, alpha B
814459	Weakly similar to unnamed protein product [H.sapiens]
212542	Homo sapiens mRNA; cDNA DKFZp586J2118 (from clon
2273304	KIAA0644 gene product

Figure 13. **Genes downregulated by hypoxia after 24 hours.**

CYCLIC GENES, + - + - + - + -

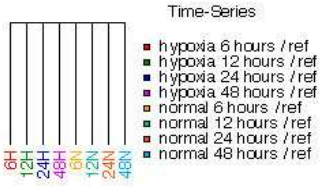


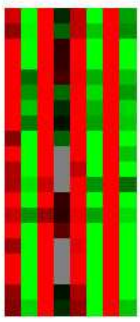


ID	Name
2213824	Protease inhibitor 3, skin-derived (SKALP)
2273362	Homo sapiens mRNA; cDNA DKFZp586B0220 (from clo
462989	Homo sapiens cDNA FLJ31303 fis, clone LIVER1000082
2312470	Differentially expressed in hematopoietic lineages
2248368	**Serum amyloid A2**
741497	Lipocalin 2 (oncogene 24p3)
323028	Homo sapiens cDNA: FLJ23037 fis, clone LNG02036, hig
1917449	**Serum amyloid A4, constitutive**
161456	**Serum amyloid A1**
741977	B-factor, properdin
446888	ESTs
1476214	Syntaphilin
279018	Multiple cluster assignments (2)
448286	ESTs
325247	ESTs
435948	Aldo-keto reductase family 1, member B1 (aldose reducta
503051	ESTs
840708	Hypothetical protein MGC5618
1556433	**GRO3 oncogene**
80186	No cluster assignment
324437	**GRO1 oncogene (melanoma growth stimulating activity,**
845355	Cathepsin C
73527	**Colony stimulating factor 1 (macrophage)**
121637	Hypothetical protein FLJ23231
1536014	V-yes-1 Yamaguchi sarcoma viral related oncogene hom
727147	**Serine (or cysteine) proteinase inhibitor, clade B ...**
2557533	**Serine (or cysteine) proteinase inhibitor, clade B ...**
470393	Matrix metalloproteinase 7 (matrilysin, uterine)
3545760	
346860	Hypothetical protein MGC5618
1569876	ESTs
768561	**Small inducible cytokine A2 (monocyte chemotactic ...**
1874367	**Small inducible cytokine subfamily A (Cys-Cys), membe**
1620712	Fibulin 5

Figure 14. **Genes fluctuating over time.**

CYCLIC GENES, + - + 0 + - + -





ID	Name
504927	Epithelial protein up-regulated in carcinoma, membrane .
340734	Nuclear factor of kappa light polypeptide gene enhancer
2508563	**Tumor necrosis factor, alpha-induced protein 3**
141806	**Tumor necrosis factor, alpha-induced protein 3**
293413	Intercellular adhesion molecule 1 (CD54), human ...
1569876	ESTs
201890	Baculoviral IAP repeat-containing 3
127032	Baculoviral IAP repeat-containing 3
788136	Phosphodiesterase 4B, cAMP-specific (dunce ...
562729	S100 calcium-binding protein A8 (calgranulin A)
815214	Chitinase 3-like 2
1475574	**Colony stimulating factor 1 (macrophage)**
549933	**Interleukin 8**
47359	**Endothelin 1**
770670	**Tumor necrosis factor, alpha-induced protein 3**
120544	Vanin 3
854338	Chitinase 3-like 2
446927	**Tumor necrosis factor (TNF superfamily, member 2)**
66437	Lipopolysaccharide-binding protein
84730	Lipopolysaccharide-binding protein

Figure 15. **Genes fluctuating over time.**

APPENDIX B

DNA LABELING FOR FLOW CYTOMETRY

500 ml VINDELOV'S PROPIDIUM IODIDE SOLUTION

Tris (hydroxy-methyl-Amino-methan) pH 7.6	0.21 g
Propidium iodide	0.025 g
Nonidet P40	0.5 ml
RNase	9.46 mg
NaCl	0.29 g

PHENOL-CHLOROFORM EXTRACTION

- 2/10 volumes of chloroform was added to the TRIzol-cell suspension.
- The sample was centrifuged at 12,000 x g for 15 minutes at 4ºC.
- The supernatant was added to a 15 ml polypropylene tube.
- 1.0 volumes of 70 % ethanol was added drop-wise to the supernatant while vortexing.

RNeasy KIT PURIFICATION

All centrifugations were performed at 2880 x g at room temperature for 5 minutes.

- The supernatant from the previous chloroform-phenol extraction was added to an RNeasy midi column, seated in a 15 ml centrifuge tube and centrifuged.
- The flow-through was centrifuged again to increase the amount of RNA binding to the column matrix.
- The flow-through was discarded and 4 ml of RW1 buffer (contains GITC and ethanol) was added.
- The columns were centrifuged and washed by 2.5 ml of RPE buffer.
- The columns were centrifuged and the washing step was repeated.
- After the last washing step the columns were centrifuged for 10 minutes to ensure that the column matrix was dry.
- The columns were put in fresh 15 ml tubes and eluted by adding 250 µl of RNase free
- H_2O.
- After 1 minute, the columns were centrifuged for 5 minutes.
- The elution step was repeated to increase the RNA yield and the columns were centrifuged for 10 minutes.

The quantity and quality of the RNA was measured spectrophotometrically.

REVERSE TRANSCRIPTION GENERATING AMINOALLYL-MODIFIED cDNA

- The two samples of RNA from time point 0 were pooled into one sample.
- 30 µg of RNA of each sample was concentrated in a speedvac yielding the final volume of 8 µl.
- 3 µl of an Anchored (dT) primer was added to the RNA
- The samples were incubated 5 minutes at 70ºC.
- The samples were cooled to room temperature for 10 minutes for the primer to anneal
- For the reverse transcription (RT) reaction the following reagents were added;

5xCyScript Buffer	4 µl
0.1M DTT	2 µl
Nucleotid Mix	1 µl
Aminoallyl-dUTP	1 µl
CyScript RT	1 µl

- The samples were incubated at 42ºC for 1,5 hrs.
- For degradation of the remaining RNA 2 µl of 2.5 M NaOH was added.
- The samples were incubated at 37ºC for 15minutes.
- 10 µl of 2M HEPES free acid was added.

PURIFICATION OF AMINOALLYL-MODIFIED cDNA BY ETHANOL PRECIPITATION

- 3 µl 3 M NaAc (pH 5.2) and 75 µl 100% ethanol was added to each reaction.
- The reactions were incubated on dry ice for 30 minutes.
- The reactions were centrifuged at 13 000 rpm for 30 minutes at 4ºC and put on ice.
- The supernatant was removed and 1 ml 70% ethanol was added.
- The reactions was centrifuged at 13 000 rpm for 15 minutes at room temperature.
- The supernatant was removed and the pellets were air dried at 37ºC and resuspended in 15 µl RNase free dH_2O.
- The samples were incubated 5 minutes in room temperature to resuspend the pellet.
- The cDNA was stored overnight at -20ºC.

CY-DYE COUPLING OF THE cDNA

Cy5 was used to label the reference cDNA from time point 0.
Cy3 was used to label the other samples of cDNA;
Each time point (6, 12, 24 and 48 hrs) was represented by a control and a hypoxic sample yielding 8 samples labeled with Cy3 and 8 reference samples.
All reactions were kept in the dark as much as possible not to degrade the CyDyes.

- Cy3 and Cy5 were resuspended in 15 µl 0.1 M $NaHCO_3$ (pH 9.0).
- The resuspended CyDye solutions were added to the samples.
- The samples were incubated for 1 hr at room temperature, in the dark.
- 15 µl 4M Hydoxylamine was added.
- The samples were incubated for 15 minutes at room temperature, in the dark.

PURIFICATION OF THE LABELED CDNA

- One CyScribe GFX column was placed into a collection tube per sample.
- 500 µl of capture buffer was added to each column.
- The samples were added to the columns.
- The columns were centrifuged at 13 800 x g for 30 seconds.
- The flow-through was discarded.
- 600 µl of wash buffer was added to each column and centrifuged as above.
- The flow-through was discarded.
- The wash step was repeated two more times.
- After the final wash the tubes were centrifuged for an additional 10 s at 13 800 x g to make

sure the columns were dry.
- Each column was placed into a fresh 1.5 ml eppendorf tube and 60 µl of pre-warmed elution buffer was added to each column. Pre-warming the elution buffer was performed at 65ºC to increase the cDNA yield.
- The columns were incubated for 5 minutes followed by centrifugation at 13 800 x g, for 1 minute.
- The elution step was repeated once.
- The reference cDNA (from time point 0) was pooled together with each sample cDNA.

FINAL PREPARATION OF THE PROBES FOR THE HYBRIDIZATION

- The block mix was prepared and added to each hybridization reaction:

  (1 hybridization)
  3.0 µl Poly dA (4 mg/ml)
  1.5 µl Yeast tRNA (4 mg/ml)
  20 µl Cot-1 DNA (20 mg/ml)

- The cDNA was dried in a speedvac until no liquid remained.
- The samples were resuspended in 40 µl DIG-Easy/1% BSA and incubated at 100ºC for 2 minutes and then at 37ºC for 30 minutes.

PREHYBRIDIZATION OF THE ARRAY SLIDES

All wash solutions were kept in Coplin jars.

- Pronto!™ Universal Pre-Soak Solution and Pronto! Universal Pre-Hybridization Solution where heated to 42ºC for 30 minutes.
- A Sodium Borohydride Pre-Soak Tablet was added to 100 ml of 42ºC Universal Pre-soak Solution. The tablet was dissolved completely.
- The slides were immersed in the pre-soak solution and incubated at 42ºC for 20 minutes.
- The slides were incubated in preheated prehybsolution (5x SSC, 0.1% SDS, 1%BSA) at 42ºC for 1 hour.
- The slides were transferred to Wash Solution 2 and incubated at room temperature for 30 seconds.
- The previous step was repeated two times.
- The slides were transferred to the 42ºC pre-hybridization solution and incubated for 15 minutes.
- The slides were put in Wash Solution 2 and incubated at room temperature for 1 minute.
- The slides were transferred to Wash Solution 3 and incubated at room temperature for 30 seconds.
- The previous step was repeated two times.
- The slides were dried by centrifugation at 500 x g for 2 minutes.

HYBRIDIZATION

8 hybridizations were performed as described in the Materials and Methods section.

- The probes were added to coverslips and flipped onto the array slides. The array slides were

put in a Corning hyb-chamber and 12 µl of dH_2O was added to prevent dehydration.
- The slides were allowed to hybridize for 18 hours at 42°C.

WASHING THE SLIDES

All wash solutions were kept in Coplin jars.

- The slides were collected and put into Wash 1 (2x SSC / 0.1% SDS) for removal of the coverslips.
- The slides were transferred to a new jar containing Wash 1 for 4 minutes.
- The slides were put into Wash 2 (1x SSC) for 4 minutes.
- The slides were put into Wash 3 (0.1x SSC) for 2 minutes.
- The slides were put into Wash 4 (0.06x SSC) for 2 minutes.
- The slides were dried by centrifugation at 500 x g, for 2 minutes at room temperature.