Time- and oxygen-dependent expression and regulation of NDRG1 in human brain cancer cells

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Abstract. N-myc downstream-regulated gene 1 (NDRG1) is a tumor suppressor with the potential to suppress metastasis, invasion and migration of cancer cells. It is regulated under stress conditions such as starvation or hypoxia. NDRG1 regulation is both induced and controlled by HIF-1α-dependent and -independent pathways under hypoxic conditions. However, there are profound differences in the way NDRG1 expression is regulated by HIF-1α and other transcription factors. Therefore, we aimed to define the time-dependent pattern of NDRG1 mRNA and protein expression in human glioblastoma cell lines in extreme hypoxia and after re-oxygenation as well as under normoxic conditions. Furthermore, we ascribe the regulation of NDRG1 to the transcription factors HIF-1α, SP1, CEBPα, YB-1 and Smad7 in a time-dependent manner. The human malignant glioma cell lines U87-MG, U373 and GaMG were cultured for 1, 6 and 24 h under hypoxic (0.1% O_2) conditions and then they were re-oxygenated. The mRNA expression of NDRG1, HIF-1α SP1, CEBPα, YB-1 and Smad7 was measured using semi-quantitative RT-PCR analysis. Their protein expression was analyzed using western blotting. Our experiments revealed that long-term (24 h), but not short-term hypoxia led to the induction of NDRG1 expression in human glioma cell lines. NDRG1 expression was found to correlate with the protein expression of HIF-1α, SP1, CEBPα, YB-1 and Smad7. The present study suggests for the first time that SP1 regulates NDRG1 expression in glioma cells under hypoxia in a time-dependent manner along with HIF-1α, CEBPα, YB-1 and Smad7. These molecules, each separately or in combination, may possess the potential to become target molecules for antitumor therapeutic approaches particularly in human brain tumors.

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Introduction

Tumor marker genes earn their functional importance from the fact that they play an essential role during disease development. Thus, their detection is fundamental for outcome monitoring and targeted treatment. Different types of tumor marker genes have been proposed including prognostic (1), diagnostic (2), predictive (3), pharmacodynamic (4) and recurrence (5) markers. In different tumor types, reduced oxygen supply to the tumor microenvironment, namely hypoxia, plays an important role during disease development to more severe stages (6). Hypoxia-inducible factor-1α (HIF-1α) is known to play an essential role in the regulation of genes induced or upregulated by the hypoxic tumor microenvironment (7,8). The cytoplasmic protein N-myc downstream-regulated gene 1 (NDRG1), also known as CAP43, non-race specific disease specific protein 1 (NDR1), differentiation-related gene 1 protein (Drg-1), nickel-specific induction protein (Cap43), protein regulated by OXYgen-1 (PROXY1) and NMSL protein, belong to the α/β-hydrolase superfamily (9). NDRG1 is involved in a diversity of cellular characteristics such as specific stress responses, hormone responses, cell growth and differentiation. NDRG1 gene mutations cause the neuropathy Charcot-Marie-Tooth disease type 4D (10). Its expression may also possess the potential to be used as a prognostic factor for some types of cancer (11). Cell differentiation signals upregulate NDRG1 and result in metastasis suppression (9). However, observations regarding the role of NDRG1 in human cancer development are controversial. In hepatocellular carcinoma (HCC) upregulation of NDRG1 has been shown to correlate with tumor aggressiveness and patient survival (11); in colon cancer cell lines NDRG1 downregulation was observed and its overexpression led to induction of cell differentiation (12). Moreover, a low NDRG1 protein expression level was found to correlate

with unfavorable patient prognosis in glioma (13), colorectal cancer (14), esophageal squamous cell carcinoma (15), pancreatic ductal adenocarcinoma (16), prostate (17,18) and breast cancer (19).

Under hypoxia, accumulation of HIF-1α and its translocation to the nucleus along with the co-activator CBP/p300 results in its binding to hypoxia-responsive elements (HREs) of target gene promoters and subsequent regulation of their transcription (22,23). Studies on the NDRG1 promoter identified HREs in the upstream promoter region and suggest regulation of NDRG1 by HIF-1α (24). However, these studies have revealed controversial results for the manner of NDRG1 regulation by HIF-1α under hypoxia. Various of these studies reported a marked upregulation of the NDRG1 expression level under hypoxic conditions, whereas others have shown downregulated NDRG1 under the same conditions (8,13-15,18,19,25). Consequently, the regulation of NDRG1 under hypoxia is highly complex and its cellular function is still a matter of debate. However, it is established that besides HIF-1α several other transcription factors involved in tumorigenesis are implicated in its regulation (8,20,21). Thus, it is important to clearly define the role of NDRG1 during carcinogenesis as well as the regulatory events steering this process.

It has been shown that different signal transduction pathways are modulated and controlled by different factors such as cytokines and growth factors or environmental conditions such as hypoxia for example (26). Transforming growth factor-β1 (TGF-β1) is one such signaling protein. Pathways involving Smad family members are also important regulators (27). Such proteins are crucial for the pathophysiological regulation and involved in the development of various diseases including fibrosis (28) and cirrhosis, leading to HCCs (29). Particularly, one member of the Smad family, Smad7, abrogates the TGF-β1 signaling pathway by initiating a negative feedback loop (30). Such a regulation may be involved in the process of cancer manifestation as seen for HCC (31). Smad7 is regulated by different pathways (32). One of these involves the transcription factor YB-1, a member of the cold shock family of proteins (30).

Specific transcription factor 1 (SP1) is another protein involved in the regulation of many cancer-related genes (33). In addition, it is involved in the active regulation of vascular endothelial growth factor (VEGF) which is another HIF-1α regulated hypoxia gene (34). Since the HIF-1α promoter contains SP1-specific transcription elements (35), SP1 may also be an important factor in NDRG1 regulation.

The transcription factor CCAAT enhancer binding protein α (CEBPα), which belongs to the BLZIP family (36), plays an important role in governing metabolic processes relevant for disease development including cancer (37). Although HIF-1α is well recognized as a main regulator during hypoxia (38), the role of other transcription factors during hypoxic conditions in the tumor microenvironment have not yet been clearly established. Therefore, we aimed to examine NDRG1 protein and mRNA expression under different oxygenation conditions in human glioblastoma multiforme (GBM) cell lines to gain further insight into its biological role. In addition, the expression levels of potential NDRG1 gene-regulating transcription factors HIF-1α, SP1, CEBPα Smad7 and YB-1 were investigated. At least to a certain extent, we planned to address, whether these factors may have the potential to serve as tumor markers.

Materials and methods

Cell lines, cell culture and hypoxia treatment. The human malignant glioma cell lines U87-MG and U373 were originally purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). GaMG is an established cell line from a patient with GBM (Gade Institute, University Bergen, Bergen, Norway) (39). Cell lines were grown on glass Petri dishes in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 μg/ml) and 2 mM L-glutamine in a humidified incubator with 5% CO_2 at 37˚C. For hypoxia treatment, the cells were exposed to 0.1% O_2 for 1, 6 and 24 h in a Ruskinn hypoxic workstation (Ruskinn Technology Ltd., Cincinnati, OH, USA) as previously described (8). Cells incubated under aerobic conditions served as the negative control; cells treated with 100 μM of the chelating agent desferrioxamine (DFO) under aerobic conditions served as the positive control.

Nuclear extract preparation. Nuclear extracts were prepared according to previously described protocols with minor modifications (8,40). A total of $5x10^6$ cells were used for each experiment. The cells were scratched-off the Petri dishes using 10 ml phosphate-buffered saline (PBS) and a pellet was obtained by centrifugation. The supernatant was discarded. Subsequently, the cells were transferred into a pre-chilled micro-centrifuge tube, re-suspended in 1 ml PBS and centrifuged at 14,000 x g for 45 sec at 4˚C. The cells were then gently re-suspended in 400 μl ice cold hypotonic buffer [10 μM/l HEPES pH 7.9, 10 μM/l KCl, 0.1 μM/l ethylenediaminetetraacetic acid (EDTA), 0.1 μM/l ethylene glycol tetraacetic acid (EGTA), 1 μM/l phenylmethylsulfonyl fluoride (PMSF), 10 μl complete protease inhibitor cocktail (Roche, Mannheim, Germany) and 1 μmol/l dithiothreitol (DTT)] by pipetting up and down several times, and then incubated on ice for 15 min. The cells were lysed by adding 25 μl of 10% NP40 with successive vortexing for 10 sec at the highest setting. The homogenate was centrifuged for 10 min at 3,000 x g at 4˚C. The supernatant, containing the cytoplasmic fraction, was transferred and saved. The pellet, containing the nuclear extract, was re-suspended in 50 μl complete cell extraction buffer [20 μM/l HEPES pH 7.9, 0.4 M/l NaCl, 1 μM/l EDTA, 1 μM/l EGTA, 1 μM/l PMSF and 0.1 μl protease inhibitor cocktail (Roche)] by shaking it for 20 min at 4˚C in a tube shaker followed by centrifugation at 14,000 x g and 4˚C for 5 min. The supernatant was transferred to a clean micro-centrifuge tube and stored at -80˚C. The protein concentration was quantitated using the Bradford method protein Quantitation Assay kit 5000001 (Bio-Rad, Munich, Germany).

Whole-cell lysate preparation and western blotting. Whole-cell lysates were prepared with 0.1 ml RIPA buffer (Amresco, Vienna, Austria), 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors pepstatin A 1.4 μM, aprotinin 0.15 μM and leupeptin 2.3 μM and 100 μM PMSF (all from Sigma-Aldrich, Munich, Germany). Phosphatase inhibitor mix

Table I. Examined genes and primers used.

	Gene	Gene Bank accession no.	Forward primer (FP)	Reverse primer (RP)	PCR product size (bp)
1	SP1	NM138473.2	5'-ATGGCAAGACCTCTCACCTG-3'	5'-TCTCTTGGACCCATGCTACC-3'	526
2	Smad7	NM_005904.2	5'-CTCGGTGCTCAAGAAACTGA-3'	5'-AATCCATCGGGTATCTGGAG-3'	362
3	CEBPα	NM_004364.2	5'-AACCTTGTGCCTTGGAAATG-3'	5'-CCCTATGTTTCCACCCCTTT-3'	246
4	YB-1	NM_004559.3	5'-GGAGATGAGACCCAAGGTCA-3'	5'-GGTGCTTGCAGTTTGTTGAC-3'	551
5	Egr-1	NM_001964.2	5'-AGCTGGAGGAGATGATGCTG-3'	5'-ACAAGGTGTTGCCACTGTT-3'	348
6	HIF-1α	NM_001530.2	5'-TTACAGCAGCCAGACGATCA-3'	5'-CCCTGCAGTAGGTTTCTGCT-3'	233
7	NDRG1	NM_006096.3	5'-CTCTGTTCACGTCACGCTGT-3'	5'-CTCCACCATCTCAGGGTTGT-3'	593
8	β-actin	NM_001101.3	5'-CGTGCGTGACATTAAGGAGA-3'	5'-CACCTTCACCGTTCCAGTTT'-3	668

The analyzed genes, accession numbers, primer sequences and lengths of PCR products are listed.

(Sigma-Aldrich) was added. The lysates were transferred to micro-centrifuge tubes, followed by 30 min incubation on ice. Subsequently, cell lysates were cleared by centrifugation at 15,000 x g for 12 min at 4˚C.

Western blotting was performed using 20 μg of protein lysates as previously described (40,41). The protein lysates were size-fractionated on 8% polyacrylamide gels (NuPAGE; Life Technologies Carlsbad, CA, USA) by electrophoresis, transferred onto nitrocellulose membranes (Protran BA85; Schleicher & Schuell, Dassel, Germany), and incubated with antibodies directed against HIF-1α (BD Biosciences, Heidelberg, Germany), CEBPα (Abcam Plc., Cambridge, UK), Egr1, SP1 (both from Santacruz Biotech, Heidelberg, Germany), NDRG1, YB-1, MADH7 (all from Abcam Plc.), β-actin and β-tubulin antibodies (both from Sigma-Aldrich). Bound antibodies were detected by developing the membrane using the ECL Plus western blotting detection system for 5 min with subsequent development of the Hyperfilm (both from Amersham Biosciences, Cambridge, UK).

Total RNA isolation and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was isolated from GBM cell lines as previously described (40,41). The RNA (1-5 μg) was reverse transcribed to cDNA using the RevertAid Reverse Transcription kit 1691 (Thermo Fisher Scientific GmbH, Schwerte, Germany) in a 20 μl reaction mixture for 1 h at 42˚C according to the manufacturer's instructions. RT-PCR analysis then was performed utilizing PCR systems and reagents from Promega™ (Mannheim, Germany) according to the manufacturer's instructions. The primers were designed in flanking exons with Primer3 software (available online http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi) (Table I). The PCR products were separated on 1% agarose gels (Sigma-Aldrich) and visualized by staining with 0.07 μg/ml ethidium bromide (Bio-Rad).

Densitometric evaluation and statistical analysis. Western blotting band densities and signal strength of RT-PCR were analyzed with 1D Kodak Image Analysis Software (8). DNA or protein amounts were calculated as previously described (21). Statistical data analysis was performed using SPSS 15.0 (SPSS, Inc., Chicago, IL, USA). All experiments were conducted as triplets. Differences between groups were analyzed with either the Student's-test (unpaired, two-tailed) or the Mann-Whitney U test. Statistical significance was considered at $p<0.05$.

Results

Time- and oxygen concentration-dependent protein expression of NDRG1 and its potential transcription factors. Long-term but not short-term hypoxia leads to the induction of NDRG1 in human glioma cell lines. Previous studies have shown that such a change is associated with tumor progression and an unfavorable prognosis of glioma patients (8). Since hypoxia plays an important role in the regulation of NDRG1 expression, we examined the NDRG1 mRNA and protein expression levels and its regulatory pathways under extreme hypoxia and re-oxygenation in the human glioma cell lines U87-MG, U373 and GaMG. We assumed that the hypoxia-induced transcription factors HIF-1α, CEBPα, Egr-1, SP1, YB-1 and Smad7 may be involved in NDRG1 regulation. Therefore, we examined the protein levels of NDRG1 and of the above-mentioned transcription factors under normoxia (21% O_2) and under extreme hypoxic conditions (0.1% O_2), which were applied for 1, 6 and 24 h. An additional analysis of protein levels was conducted 24 and 48 h, after re-oxygenation. NDRG1 protein expression was not detectable under normoxic or hypoxic conditions for 1 h in all cell lines analyzed (Fig. 1). Only U87-MG cells exhibited low NDRG1 protein expression under these conditions. However, this cell line exhibited an increase in NDRG1 protein expression after re-oxygenation (Fig. 1B). U373 cells displayed moderate NDRG1 protein expression after 6 h of hypoxia (Fig. 1C), whereas NDRG1 expression was increased in the GaMG cells under extended hypoxia of 24 h (Fig. 1A). In all cell lines, high NDRG1 expression remained stable after re-oxygenation (Fig. 1). Among the analyzed transcription factors, Smad7 displayed a notably alternating response to hypoxia and re-oxygenation. The Smad7 protein expression was undetectable under normoxia, very low after 1 h of 0.1% O_2, disappeared again after 6 h hypoxia and then increased to the highest concentration after 24 h hypoxia in GaMG cells (Fig. 1A). Re-oxygenation after 24 h of hypoxia led to a

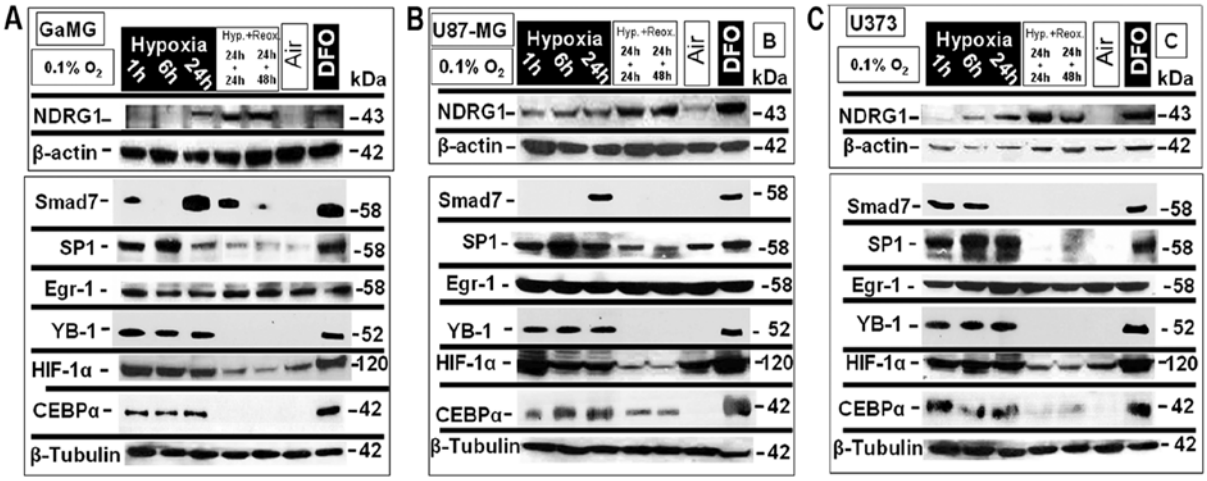

Figure 1. *In vitro* protein expression analysis in glioblastoma multiforme (GBM) cells under different oxygenation conditions. The GBM cell lines, GaMG, U87-MG and U373, were exposed to extreme hypoxic oxygenation conditions (0.1% O_2) for up to 24 h and then re-oxygenated for 24 h and 48 h. Normoxia and treatment with desferoxamine (DFO; 100 μM) for 24 h served as negative and positive controls, respectively. The protein expression of NDRG1 and of the transcription factors Smad7, SP1, Egr-1, YB-1, HIF-1α and CEBPα was analyzed by western blotting. A clear O_2 concentration- and time-dependent expression regulation and also cell line-specific differences were observed. (A) In GaMG, NDRG1 protein expression increase was only significant after 24 h and at the highest expression level and relatively stable upon re-oxygenation for a period of up to 48 h after 24 h of hypoxia. Smad7 protein expression (3rd row from above) pattern displayed a significant protein expression level increase after only 1 h of exposure to extreme hypoxia which decreased to the basal expression level after 6 h of hypoxia and again increased to a maximum level after 24 h of hypoxia, while, re-oxygenation resulted in a significant decrease in the expression level. SP-1 expression pattern (4th row from above) showed a significant increase in the expression level after only 1 h of extreme hypoxia that reached its maximum after 6 h of hypoxia and decreased to expression levels below that of the previous ones, but still higher than the basal expression level and of 24 h of hypoxia, while re-oxygenation up to 48 h after 24 h of hypoxia resulted in a decrease to a lower level slightly higher than those at basal expression level. Egr-1 expression level was stable in response to the different oxygenation conditions. YB-1 expression (6th row from above) was significantly increased after 1 h of hypoxia and was relatively stable up to 24 h of extreme hypoxia; while re-oxygenation for 24 and 48 h, respectively after 24 h of hypoxia resulted in the decrease to an expression level similar to the basal expression level. HIF-1α protein expression level (7th row from above) increased after 1 h of extreme hypoxic exposure and was stable up to 24 h of extreme hypoxia and its level decreased to a similar pattern as the basal expression level under normoxic conditions. CEBPα showed an expression pattern similar to that of YB-1 expression with a lower protein level expressed. (B) In U87-MG, NDRG1 expression increased significantly after 1 h of extreme hypoxia and continued to increase in its expression level up to 24 h of hypoxia and 24 and 48 h of re-oxygenation after 24 h of hypoxia and returned to basal expression level under nomoxic oxygenation conditions. Smad7 expression increased only after 24 h of hypoxia reaching its maximum. SP-1 expression pattern was similar to that in GaMG, but with a higher protein expression level. Egr-1 expression level was stable in response to the different oxygenation conditions examined. YB-1 and HIF-1α expression was displayed in a comparable pattern as in GaMG, with a higher protein level of HIF-1α, while CEBPα expression increased significantly only after 1 h of hypoxia and was at a stable level for up to 24 h of hypoxia but it decreased significantly upon re-oxygenation after 24 h of hypoxia. (C) In U373, NDRG1 expression was significantly increased after 1 h of extreme hypoxia and maintained an increase up to 24 h of hypoxia and 24 and 48 h of re-oxygenation. Smad7 expression level increased after only 1 h of hypoxia and was stable at 6 h of hypoxia but started to decrease to basal expression level after 24 h of hypoxia. SP-1 was expressed at a significant level after only 1 h of hypoxia and increased in its expression level gradually until 24 h, followed by a decline to basal level after 24 h of re-oxygenation and a relative increase after 48 h of re-oxygenation. Egr-1 again showed no change in its displayed pattern in response to the alternating oxygenation conditions. YB-1 and HIF-1α expression patterns were the same as the corresponding protein as was the case in U87-MG with a higher protein expression rate for HIF-1α, and CEBPα expression level increased significantly after 1 h of extreme hypoxic exposition and decreased after 6 h and increased again after 24 h of extreme hypoxia to its maximum level, and decreased again upon re-oxygenation to its basic expression level. One representative experiment out of three independent repetitions is shown. The housekeeping genes β-actin and β-tubulin served as loading controls.

decrease in the Smad7 protein level. In the U87-MG cells, no Smad7 protein expression was detectable, except after 24 h of hypoxia, which led to moderate expression (Fig. 1A). Notably, U373 cells displayed a different expression pattern. Smad7 expression increased after 1 h of hypoxia with a moderate decrease after 6 h and a complete disappearance after 24 h (Fig. 1C). Under re-oxygenation or normoxia no Smad7 expression was detectable in this cell line (Fig. 1C). Hypoxic conditions caused an early increase of SP1 protein already after 1 h in all cell lines, although the magnitude varied from strong (U373 and U87-MG) to moderate (GaMG) (Fig. 1). Moreover, the SP1 protein expression continued to increase and reached a peak after 6 h, to be reduced again after 24 h of hypoxia. After re-oxygenation SP1 expression disappeared in the U373 cells, was low in GaMG and moderate in U87-MG cells, resembling the normoxic expression levels of these cell lines (Fig. 1C).

All three examined cells lines expressed Egr-1 protein with no alterations caused by hypoxia or re-oxygenation (Fig. 1). YB-1 was not expressed under normoxia or after re-oxygenation, but hypoxic conditions induced YB-1 protein expression already after 1 h, which remained stable for at least 24 h in all three cell lines (Fig. 1). Hypoxic conditions led to an early increase in HIF-1α protein expression in all three cell lines. Re-oxygenation then caused a considerable decrease of protein expression, but not in its complete disappearance (Fig. 1).

CEBPα displayed a similar pattern under hypoxic conditions with higher expression levels in U87-MG and U373 cells compared to GaMG. Re-oxygenation led to the disappearance of detectable CEBPα protein in GaMG cells, while in U373 and U87-MG residual protein still was detectable with highest concentrations in the U87-MG cells (Fig. 1B). Desferoxamine (DFO) was used as a control and significantly induced the protein expression of all proteins analyzed (Fig. 1).

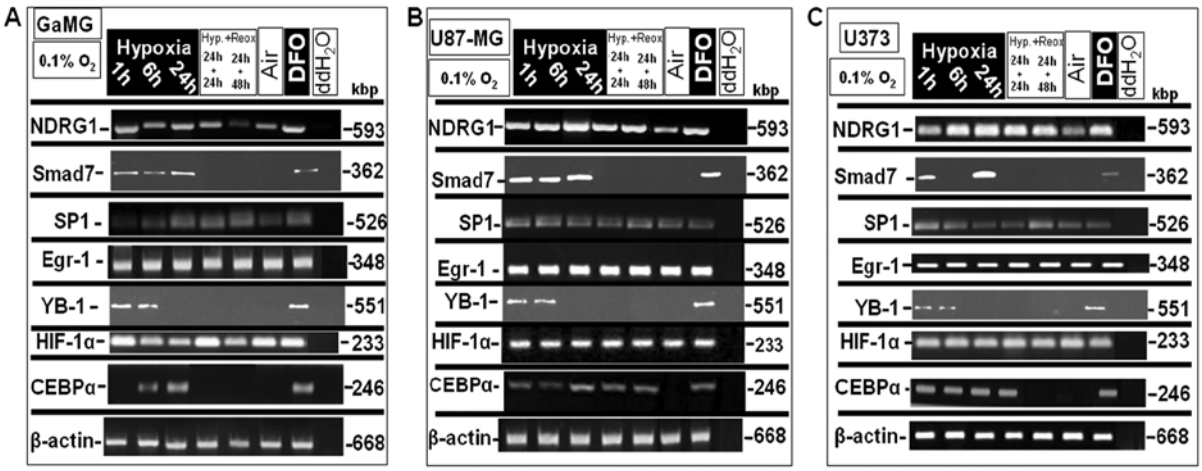

Figure 2. *In vitro* mRNA expression analysis in glioblastoma multiforme (GBM) cells under different oxygenation conditions. The GBM cell lines GaMG, U87-MG and U373 were exposed to extreme hypoxic oxygenation conditions (0.1% O_2) for up to 24 h and then were re-oxygenated for 24 and 48 h. Normoxia and treatment with 100 μM DFO for 24 h served as negative and positive controls, respectively. mRNA-lysates were then analyzed by semi-quantitative RT-PCR for the expression of NDRG1 and of the transcription factors Smad7, SP1, Egr-1, YB-1, HIF-1α and CEBP-α. (A) In GaMG, NDRG1 mRNA expression (1st row, left column) increased after just 1 h of extreme hypoxic exposure and remained at a stable level up to 24 h, and 24 h of re-oxygenation after 24 h of hypoxia, but declined to an expression level nearly to that expressed under normoxia. Smad7 mRNA expression level (2nd row) increased after 1 h of hypoxia and was stable up to 24 h of hypoxia and declined upon re-oxygenation after 24 h to the basal expression level. SP-1 expression (3rd row), increased to its maximum level after 24 h of hypoxia and was stable upon re-oxygenation for 24 and 48 h, respectively, after 24 h of hypoxia. Egr-1 expression (4th row) was at a stable level under the different conditions examined. YB-1 expression level (5th row, left column) reached its maximum level after just 1 h of hypoxic exposition and declined to an expression level similar to that noted under normoxia. HIF-1α mRNA (6th row) was stable under the different aeration conditions examined. CEBPα expression (7th row) increased significantly after 6 h of hypoxia reaching its maximum after 24 h and declined to the basal expression level upon re-oxygenation. (B) In U87-MG, NDRG1 (1st row, middle column) reached its maximum mRNA expression level after only 1 h of hypoxia and was at a stable level under all examined aeration levels and declined to a basal expression level under normoxia. Smad7 mRNA expression (2nd row) behaved in a similar way as in GaMG cells with a higher expression rate. SP-1 mRNA showed a significant expression pattern (3rd row), starting with a significant increase in to a high expression level after 1 h reaching its maximum level after 6 h with a relative decline after 24 h of hypoxia and 24 h of re-oxygenation and after hypoxia and again a slight increase at 48 h of re-oxygenation after 24 h of hypoxia. Egr-1 expression (4th row) was stable with a uniform pattern during the different aeration conditions examined. YB-1 (5th row) reached its maximum expression level after just 1 h of hypoxic exposition, and was stable after 6 h and declined to a basal expression level after 24 h of hypoxia and re-oxygenation after hypoxia. HIF-1α (6th row) was expressed at a stable level under all conditions examined. CEBPα mRNA expression increased significantly after only 1 h of hypoxia, then declined to one half of the expression after 6 h of hypoxia and reached its maximum after 24 h and declined to an expression level similar to a basal expression level upon re-oxygenation after hypoxia. (C) In U373, NDRG1 mRNA (1st row), reached its maximum level after only 6 h of hypoxia and was stable under the different aeration conditions examined. Smad7 mRNA (2nd row) showed a notable expression pattern, showing a significant increase in the expression level after 1 h of hypoxia, that declined after 6 h and increased again to its maximum expression level after 24 h. Re-oxygenation after hypoxia resulted in minimization to a basal expression level. SP-1 expression (3rd row) was also interesting as it reached its maximum level after only 1 h of hypoxia and continuing with a lower expression rate for 6 and 24 h of hypoxia and 24 h re-oxygenation after 24 h of hypoxia and reaching again the maximal expression level after 48 h of re-oxygenation after 24 h of hypoxia. Egr-1 mRNA (4th row) displayed a constant expression pattern under all oxygenation conditions examined. YB-1mRNA (5th row) reached it maximum after 1 h and was stable up to 6 h and declined after 24 h as well after re-oxygenation. HIF-1α (6th row) showed a constant expression pattern under the different oxygenation conditions examined. CEBPα mRNA (7th row), was significantly expressed at its maximum expression level after only 1 h of hypoxia which kept constant up to 24 h of hypoxia and 24 h of re-oxygenation after hypoxia and declined towards the basal expression level upon re-oxygenation for 48 h after 24 h of hypoxia. One representative experiment out of three independent repetitions is shown. The housekeeping gene β-actin served as a loading control.

Time- and oxygen concentration-dependent mRNA expression of NDRG1 and its potential transcription factors. The mRNA expression levels of CEBPα, HIF-1α, EGR-1, Smad7, YB-1 and SP1 in the human glioma cell lines U87-MG, U373 and GaMG cultivated under different oxygenation conditions are illustrated in Fig. 2.

NDRG1 mRNA was detectable under all conditions in all cell lines analyzed. However, hypoxia procured a relative increase of mRNA levels with time. Re-oxygenation led to a slight reduction after 48 h. NDRG1 mRNA expression was highest in U373 cells, followed by U87-MG and lowest in GaMG, which also was most sensitive to re-oxygenation (Fig. 2).

Smad7 mRNA expression was clearly dependent upon the oxygenation conditions. It was not detectable under normoxia or after re-oxygenation, but highly induced already after 1 h of hypoxia. Notably, Smad7 mRNA expression disappeared after 6 h of hypoxia in the U373 cells and re-appeared after 24 h to a higher level then after 1 h (Fig. 2C). U87-MG cells displayed the strongest mRNA expression and GaMG the lowest level (Fig. 2A and B).

SP1 mRNA expression also showed significant features. In U87-MG cells, SP1 mRNA was moderately expressed and without changes under all tested conditions (Fig. 2B). However, in GaMG cells there was an increase over time under hypoxia, which remained stable at a high level after re-oxygenation (Fig. 2A). In contrast, the U373 cells had increased SP1 mRNA expression noticeably after 1 h of hypoxia, which then decreased back to basal levels over 24 h. The same basal expression was visible after 24 h of hypoxia followed by 24 h of re-oxygenation. Surprisingly, 48 h re-oxygenation caused the strongest SP1 mRNA expression in this experiment (Fig. 2C).

The same Egr-1 mRNA expression strength was detectable under all conditions in all three cell lines examined (Fig. 2).

In contrast, YB-1 mRNA expression was induced by 1 and 6 h of hypoxia, but disappeared after 24 h of hypoxia. It was not detectable under normoxia or after re-oxygenation.

HIF-1α mRNA was expressed at a constant level under all oxygenation conditions in the three cell lines, which is a common expression feature of HIF-1α (Fig. 2).

CEBPα displayed a cell line-specific expression pattern. In all three cell lines there was expression under normoxia (Fig. 2). Hypoxia induced CEBPα expression. In the GaMG cells there was very low expression detectable after 1 h hypoxia, which considerably increased after 6 h and reached the highest levels after 24 h. CEBPα expression then disappeared under re-oxygenation (Fig. 2A). In contrast, the U87-MG cells exhibited increased CEBPα mRNA expression strongly already after 1 h of hypoxia, then after 6 h the cells showed a decrease in expression by 50% to increase the expression again after 24 h. The expression remained high even after re-oxygenation (Fig. 2B). In the U373 cells, there was a significant increase in CEBPα mRNA expression after 1 h of hypoxia, which remained constant over 24 h and only disappeared after 48 h of re-oxygenation (Fig. 2C).

DFO was able to significantly induce the mRNA expression of all tested factors in the three cell lines examined (Fig. 2).

Discussion

The present study showed that NDRG1 expression was associated with long-term hypoxia, but not with short-term hypoxia in human GBM cells. NDRG1 expression may be under the control of HIF-1α in addition to the other transcription factors examined in a so-called expression-regulated time point. The significant increase in NDRG1 expression in hypoxia-exposed GBM cells confirms our previous findings of NDRG1 as a hypoxia-associated molecule with overexpression in tumor cells (21). A common survival strategy of tumor cells at the beginning of hypoxia includes Pasteur-effect remission, in this case it appears that HIF-1α binds to HER sequences of hypoxia-responsive genes (42) and thereby stimulates glycolytic transporter proteins and enzyme expression (43).

The NDRG1 gene contains two HREs within its non-coding sequence (24), indicating that its regulation is HIF-1α-dependent (44). These data support our previous findings that NDRG1 expression is induced by HIF-1α in human tumor cells (8). In addition, the HIF-1α expression precedes NDRG1 expression, indicating that it may be a prerequisite for NDRG1 upregulation since it is its major transcriptional regulator (8,21,45,46). The same holds true for other HIF-1α-regulated genes such as CA9 (47). Therefore, we suggest NDRG1 as a marker for hypoxic regions within a tumor mass (8). It may also be of prognostic relevance, since the survival rate of patients without NDRG1 expression was lower than that of patients bearing NDRG1-positive cells (13). In addition, high expression level of NDRG1 does not correlate with the overall patient survival (48).

In two of the examined GBM cell lines, NDRG1 protein expression was undetectable under normoxic conditions. Although increased NDRG1 protein expression has been reported for different types of tumor cells such as human HCC and colorectal tumors (11,49), other studies showed a decrease in its expression level such as in metastasizing colon cancer cell lines (50). Low-grade astrocytoma (LGA) displayed a lower level of NDRG1 mRNA and protein expression than GBM samples from patients (8). However, another study showed that the NDRG1 mRNA and protein expression was reduced in GBM compared to normal brain tissue (13). These observations suggest that the regulation of NDRG1 may be cancer cell-specific and also dependent on the oxygenation status of the tumor tissue.

In the present study, we showed that NDRG1 mRNA expression was downregulated upon re-oxygenation, which is in agreement with a previous study concerning decreased NDRG1 mRNA expression in the breast cancer cell line MCF-7 after re-oxygenation (51). Therefore, NDRG1 is considered to be an O_2-responsive gene, linked to tumor adaptation towards re-oxygenation (51,52). However, our western blot results did not support this notion, and suggest posttranscriptional regulation of NDRG1 expression. The stability of NDRG1 after re-oxygenation following long-time exposure to hypoxic oxygenation conditions may be related to both, post transcriptional and post translational modifications. Based on our results and other research data we can postulate that a post transcriptional modification may be achieved by NDRG1 interaction with the HSP90 protein (53). The mTOR signaling pathway may also be involved, since the downstream regulator CCR4-NOT may upregulate NDRG1 (54,55). Such post transcriptional regulation may act along with post translational modification, such as e.g. sumoylation of HIF-1α. HIF-1α is destabilized and as a consequence the transcription rate of NDRG1 is reduced. In contrast, SUMO protease-1 contributes to HIF-1α stability and thereby increases the NDRG1 transcription rate (55-57). Such modifications may contribute to the modulatory process that in the end renders NDRG1 mRNA for protein synthesis.

Egr-1 displayed a constant expression pattern at the mRNA and protein level, irrespective of the tested oxygenation conditions. This suggests a constitutive functional role for this transcription factor under various physiological conditions in cancer cells (58).

In contrast, the expression of the transcription factors HIF-1α, SP1, CEBPα, YB-1 and Smad7 widely correlated with those of NDRG1. Particularly, the nuclear mRNA levels of HIF-1α, SP1 and C/EBP-α were closely correlated with those of NDRG1. It has been shown that hypoxic conditions raise the expression of these transcription factors in nuclear extracts (59), suggesting that the hypoxia-stimulated upregulation of NDRG1 may be mainly mediated by a combined effect of these factors and to a lesser extend by Smad7 and YB-1. A cooperative mechanism, as suggested in the present study, has been shown to promote the regulation of genes necessary for cell adaptation to varying microenvironments such as during starvation and hypoxia (60). Indeed, NDRG1 promoter studies revealed that a SP1 site partially is responsible for the VHL-induced suppression of NDRG1 (61,62).

The analysis of the murine NDRG1 promoter revealed an overlap between Egr-1 and SP1 binding motifs (62). Although, it has previously been shown that NDRG1 expression during hypoxia is initiated by HIF-1α (63), SP1 triggers the hypoxic promoter activation independently (64) or by interaction with HIF-1α (65). In the present study, SP1 protein expression was increased during hypoxia in the human glioma cell lines. The highest level was detected after 6 h of extreme hypoxia. Most

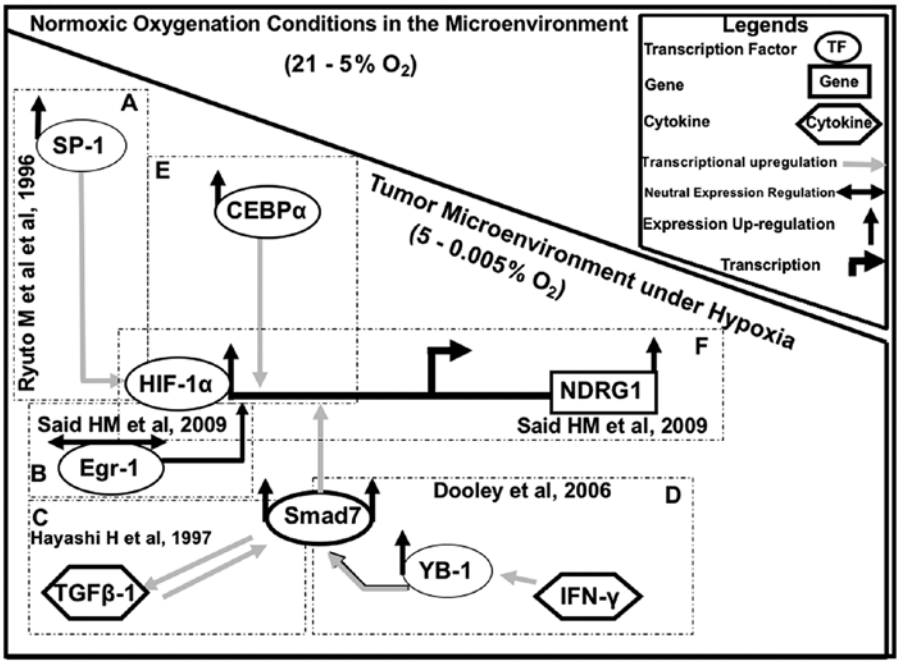

Figure 3. Proposed regulatory signaling network for hypoxia-induced gene activation in human cancer diseases. Although HIF-1α is the main regulator of hypoxia-induced genes, other signaling pathways play an important assisting role. NDRG1 is shown as an example. (A) Under hypoxia, SP1 upregulates HIF-1α and subsequently this leads to an increase in hypoxia-induced gene transcription (NDRG1). (B) Egr-1 expression remained stable despite varying oxygenation conditions. Therefore, Egr-1 may be a cofactor for the regulation of hypoxia-induced transcription. (C) TGF-β upregulates Smad7 which functions as an antagonist via a negative feedback loop. (D) IFN-γ stimulates YB-1 overexpression which then may increase Smad7 expression, and thereby provoke the overexpression of NDRG1. (E) CEBPα expression is increased in an oxygen concentration- and time-dependent manner, and thereby functions as a positive regulator for NDRG1 expression. (F) NDRG1 expression rate is increased due to the activity of the outlined network of cytokines and transcription factors.

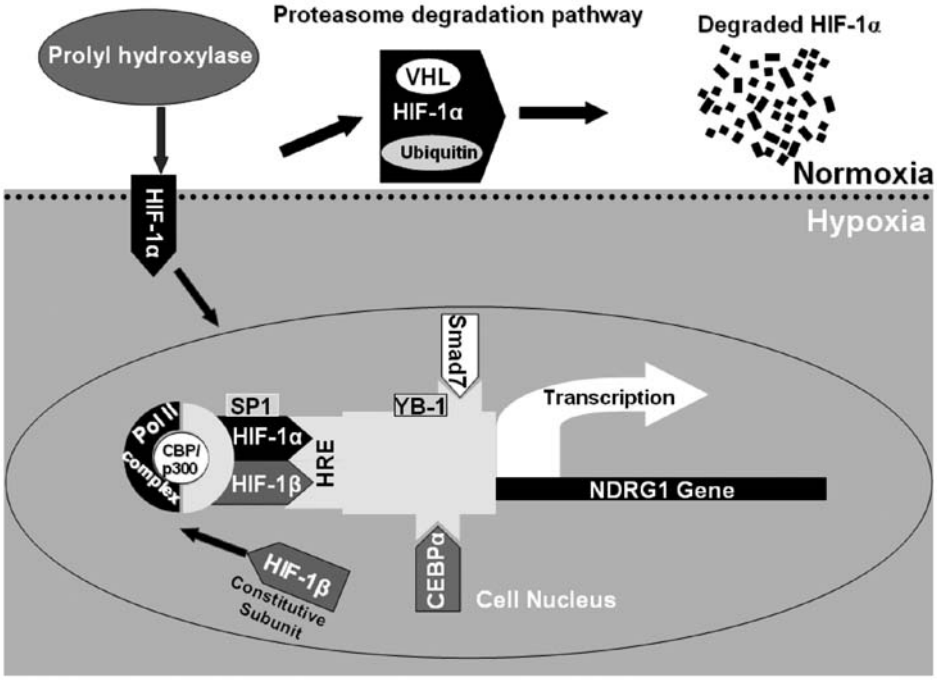

Figure 4. Summary of pathways regulating NDRG1 gene activity under different oxygenation conditions in human brain cancer cells *in vitro*. SP1, HIF-1α, CEBPα, Smad7 and YB-1 play an active role in the regulation of NDRG1 while the effect of Egr-1 is neutral. The intensity and activating potential is variable and depends on the aeration conditions and time.

of the transcription factors regulating metabolic events in cancer, including HIF-1α, have a binding site for SP1 in their promoter region (66). HIF-1α expression has been shown to be SP1-dependent in human prostate cancer cells (67), suggesting a crucial regulatory role in cancer metabolism as well as during hypoxia.

We showed an upregulation of CEBPα in human GBM cells in response to hypoxia. However, these results are not consistent with a previous study using breast cancer cells, which showed that the exposure of these cells to 1% O_2 downregulates the CEBPα production (68) and transcription (69). These differences may be explained by the severity of hypoxia, as in our experiments the GBM cells were exposed to extreme hypoxic conditions (0.1% O_2) for up to 24 h. It has been reported that the HIF-1α-bound HRE within the CEBPα promoter region is essential for CEBPα transcriptional downregulation under hypoxic conditions (62). In contrast, we observed a lack of CEBPα expression in GBM cells under normoxic conditions. CEBPα downregulation under normoxia/moderate hypoxia and its upregulation during extreme hypoxia may play a role during progression.

Different signal transduction pathways are controlled and modulated by different growth factors and environmental events such as hypoxic oxygenation conditions (26). The TGF-β pathway involves the Smad protein family (27). These proteins play an important role in triggering diseases including for example fibrosis (28) and cirrhosis, leading to HCC (29). NDRG1 overexpression could inhibit TGF-β-induced effects via reducing both Smad2 and Smad3 expression (63). However, Smad7, another member of this protein family, abrogates the TGF-β signaling pathway by a regulative negative feedback loop (30), thereby, paving the physiological conditions towards cancer disease manifestation, as HCC is an example when this negative feedback loop is hindered, while the transition to fibrosis, cirhosis and as a consequence HCC is hindered when this negative feedback loop of Smad7 remains active (31). Smad7 is regulated by different signaling pathways (32). IFNγ contributes to this regulatory scheme by inducing a member of the cold shock protein family, the transcription factor YB-1 (30,70-73) (Fig. 3).

Our results suggest that HIF-1α regulates NDRG1 expression in GBM cells in conjunction with SP1, CEBPα, YB-1 and Smad7 under different physiological conditions, whereas Egr-1 may act as a cofactor (Fig. 4). A very similar regulatory mechanism may also exist for other hypoxia-induced genes (7), such as CA9, Epo and VEGF in human brain cancer (40,64). These factors are also controlled by HIF-1α. However, other co-factors may be involved. The knowledge concerning such regulatory processes during cancer development is important for the development and application of cancer treatment modalities.

In conclusion, we showed for the first time that NDRG1 protein expression was significantly increased in GBM cells under hypoxia and that there was a correlation with the protein expression of HIF-1α, SP1, CEBPα, YB-1 and Smad7. We suggest that SP1 regulates NDRG1 in GBM cells under different oxygenation conditions in conjunction with HIF1α, CEBPα and, to a certain extent, YB-1 and Smad7. NDRG1 and its potential transcription factors therefore may represent target proteins for complementary treatment approaches. Combined, they also may serve as potential hypoxia tumor markers in gene expression analyses.

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References

1. Theunissen PH and Blaauw G: Proliferating cell nuclear antigen immunostaining and survival in cerebral astrocytoma. Histopathology 23: 75-79, 1993.
2. Kaye SB, Bagshawe KD, McElwain TJ and Peckham MJ: Brain metastases in malignant teratoma: A review of four years' experience and an assessment of the role of tumour markers. Br J Cancer 39: 217-223, 1979.
3. Molendini L, Benassi MS, Magagnoli G, Merli M, Sollazzo MR, Ragazzini P, Gamberi G, Ferrari C, Balladelli A, Bacchini P, *et al*: Prognostic significance of cyclin expression in human osteosarcoma. Int J Oncol 12: 1007-1011, 1998.
4. Nam DH, Park K, Park C, Im YH, Kim MH, Lee S, Hong SC, Shin HJ, Kim JH, Eoh W, *et al*: Intracranial inhibition of glioma cell growth by cyclooxygenase-2 inhibitor celecoxib. Oncol Rep 11: 263-268, 2004.
5. Xie SS, Tan M, Lin HY, Xu L, Shen CX, Yuan Q, Song XL and Wang CH: Overexpression of adenylate cyclase-associated protein 1 may predict brain metastasis in non-small cell lung cancer. Oncol Rep 33: 363-371, 2015.
6. Angst E, Sibold S, Tiffon C, Weimann R, Gloor B, Candinas D and Stroka D: Cellular differentiation determines the expression of the hypoxia-inducible protein NDRG1 in pancreatic cancer. Br J Cancer 95: 307-313, 2006.
7. Semenza GL: Involvement of oxygen-sensing pathways in physiologic and pathologic erythropoiesis. Blood 114: 2015-2019, 2009.
8. Said HM, Stein S, Hagemann C, Polat B, Staab A, Anacker J, Schoemig B, Theobald M, Flentje M and Vordermark D: Oxygen-dependent regulation of NDRG1 in human glioblastoma cells *in vitro* and *in vivo*. Oncol Rep 21: 237-246, 2009.
9. Ellen TP, Ke Q, Zhang P and Costa M: NDRG1, a growth and cancer related gene: Regulation of gene expression and function in normal and disease states. Carcinogenesis 29: 2-8, 2008.
10. Berger P, Young P and Suter U: Molecular cell biology of Charcot-Marie-Tooth disease. Neurogenetics 4: 1-15, 2002.
11. Chua MS, Sun H, Cheung ST, Mason V, Higgins J, Ross DT, Fan ST and So S: Overexpression of NDRG1 is an indicator of poor prognosis in hepatocellular carcinoma. Mod Pathol 20: 76-83, 2007.
12. Wangpu X, Yang X, Zhao J, Lu J, Guan S, Lu J, Kovacevic Z, Liu W, Mi L, Jin R, *et al*: The metastasis suppressor, NDRG1, inhibits 'stemness' of colorectal cancer via down-regulation of nuclear β-catenin and CD44. Oncotarget 6: 33893-33911, 2015.
13. Sun B, Chu D, Li W, Chu X, Li Y, Wei D and Li H: Decreased expression of NDRG1 in glioma is related to tumor progression and survival of patients. J Neurooncol 94: 213-219, 2009.
14. Strzelczyk B, Szulc A, Rzepko R, Kitowska A, Skokowski J, Szutowicz A and Pawelczyk T: Identification of high-risk stage II colorectal tumors by combined analysis of the *NDRG1* gene expression and the depth of tumor invasion. Ann Surg Oncol 16: 1287-1294, 2009.
15. Ando T, Ishiguro H, Kimura M, Mitsui A, Kurehara H, Sugito N, Tomoda K, Mori R, Takashima N, Ogawa R, *et al*: Decreased expression of NDRG1 is correlated with tumor progression and poor prognosis in patients with esophageal squamous cell carcinoma. Dis Esophagus 19: 454-458, 2006.
16. Maruyama Y, Ono M, Kawahara A, Yokoyama T, Basaki Y, Kage M, Aoyagi S, Kinoshita H and Kuwano M: Tumor growth suppression in pancreatic cancer by a putative metastasis suppressor gene *Cap43/NDRG1/Drg-1* through modulation of angiogenesis. Cancer Res 66: 6233-6242, 2006.
17. Nakamura K, Yasunaga Y, Segawa T, Ko D, Moul JW, Srivastava S and Rhim JS: Curcumin down-regulates AR gene expression and activation in prostate cancer cell lines. Int J Oncol 21: 825-830, 2002.

18. Bandyopadhyay S, Pai SK, Gross SC, Hirota S, Hosobe S, Miura K, Saito K, Commes T, Hayashi S, Watabe M, *et al*: The *Drg-1* gene suppresses tumor metastasis in prostate cancer. Cancer Res 63: 1731-1736, 2003.
19. Bandyopadhyay S, Pai SK, Hirota S, Hosobe S, Tsukada T, Miura K, Takano Y, Saito K, Commes T, Piquemal D, *et al*: PTEN up-regulates the tumor metastasis suppressor gene *Drg-1* in prostate and breast cancer. Cancer Res 64: 7655-7660, 2004.
20. Cangul H: Hypoxia upregulates the expression of the *NDRG1* gene leading to its overexpression in various human cancers. BMC Genet 5: 27, 2004.
21. Said HM, Polat B, Staab A, Hagemann C, Stein S, Flentje M, Theobald M, Katzer A and Vordermark D: Rapid detection of the hypoxia-regulated CA-IX and NDRG1 gene expression in different glioblastoma cells *in vitro*. Oncol Rep 20: 413-419, 2008.
22. Arany Z, Huang LE, Eckner R, Bhattacharya S, Jiang C, Goldberg MA, Bunn HF and Livingston DM: An essential role for p300/CBP in the cellular response to hypoxia. Proc Natl Acad Sci USA 93: 12969-12973, 1996.
23. Kallio PJ, Okamoto K, O'Brien S, Carrero P, Makino Y, Tanaka H and Poellinger L: Signal transduction in hypoxic cells: Inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha. EMBO J 17: 6573-6586, 1998.
24. Le NT and Richardson DR: Iron chelators with high antiproliferative activity up-regulate the expression of a growth inhibitory and metastasis suppressor gene: A link between iron metabolism and proliferation. Blood 104: 2967-2975, 2004.
25. Salnikow K, Costa M, Figg WD and Blagosklonny MV: Hyperinducibility of hypoxia-responsive genes without p53/p21-dependent checkpoint in aggressive prostate cancer. Cancer Res 60: 5630-5634, 2000.
26. Kockar F, Yildrim H, Sagkan RI, Hagemann C, Soysal Y, Anacker J, Hamza AA, Vordermark D, Flentje M and Said HM: Hypoxia and cytokines regulate carbonic anhydrase 9 expression in hepatocellular carcinoma cells in vitro. World J Clin Oncol 3: 82-91, 2012.
27. Kusanagi K, Inoue H, Ishidou Y, Mishima HK, Kawabata M and Miyazono K: Characterization of a bone morphogenetic protein-responsive Smad-binding element. Mol Biol Cell 11: 555-565, 2000.
28. Yamaguchi T, Matsuzaki K, Inokuchi R, Kawamura R, Yoshida K, Murata M, Fujisawa J, Fukushima N, Sata M, Kage M, *et al*: Phosphorylated Smad2 and Smad3 signaling: Shifting between tumor suppression and fibro-carcinogenesis in chronic hepatitis C. Hepatol Res 43: 1327-1342, 2013.
29. Neuzillet C, Tijeras-Raballand A, de Mestier L, Cros J, Faivre S and Raymond E: MEK in cancer and cancer therapy. Pharmacol Ther 141: 160-171, 2014.
30. Dooley S, Hamzavi J, Breitkopf K, Wiercinska E, Said HM, Lorenzen J, Ten Dijke P and Gressner AM: Smad7 prevents activation of hepatic stellate cells and liver fibrosis in rats. Gastroenterology 125: 178-191, 2003.
31. Del Vecchio F, Gallo F, Di Marco A, Mastroiaco V, Caianiello P, Zazzeroni F, Alesse E and Tessitore A: Bioinformatics approach to predict target genes for dysregulated microRNAs in hepatocellular carcinoma: Study on a chemically-induced HCC mouse model. BMC Bioinformatics 16: 408, 2015.
32. Liu Y, Meyer C, Müller A, Herweck F, Li Q, Müllenbach R, Mertens PR, Dooley S and Weng HL: IL-13 induces connective tissue growth factor in rat hepatic stellate cells via TGF-β-independent Smad signaling. J Immunol 187: 2814-2823, 2011.
33. Szalad A, Katakowski M, Zheng X, Jiang F and Chopp M: Transcription factor Sp1 induces ADAM17 and contributes to tumor cell invasiveness under hypoxia. J Exp Clin Cancer Res 28: 129, 2009.
34. Ryuto M, Ono M, Izumi H, Yoshida S, Weich HA, Kohno K and Kuwano M: Induction of vascular endothelial growth factor by tumor necrosis factor alpha in human glioma cells. Possible roles of SP-1. J Biol Chem 271: 28220-28228, 1996.
35. Minet E, Ernest I, Michel G, Roland I, Remacle J, Raes M and Michiels C: HIF1A gene transcription is dependent on a core promoter sequence encompassing activating and inhibiting sequences located upstream from the transcription initiation site and *cis* elements located within the 5'UTR. Biochem Biophys Res Commun 261: 534-540, 1999.
36. Osada S, Takano K, Nishihara T, Suzuki T, Muramatsu M and Imagawa M: CCAAT/enhancer-binding proteins alpha and beta interact with the silencer element in the promoter of glutathione S-transferase P gene during hepatocarcinogenesis. J Biol Chem 270: 31288-31293, 1995.
37. Darlington GJ, Wang N and Hanson RW: C/EBP alpha: A critical regulator of genes governing integrative metabolic processes. Curr Opin Genet Dev 5: 565-570, 1995.
38. Hubbi ME and Semenza GL: Regulation of cell proliferation by hypoxia-inducible factors. Am J Physiol Cell Physiol 309: C775-C782, 2015.
39. Akslen LA, Andersen KJ and Bjerkvig R: Characteristics of human and rat glioma cells grown in a defined medium. Anticancer Res 8: 797-803, 1988.
40. Said HM, Staab A, Hagemann C, Vince GH, Katzer A, Flentje M and Vordermark D: Distinct patterns of hypoxic expression of carbonic anhydrase IX (CA IX) in human malignant glioma cell lines. J Neurooncol 81: 27-38, 2007.
41. Said HM, Katzer A, Flentje M and Vordermark D: Response of the plasma hypoxia marker osteopontin to in vitro hypoxia in human tumor cells. Radiother Oncol 76: 200-205, 2005.
42. Madan A and Curtin PT: A 24-base-pair sequence 3' to the human erythropoietin gene contains a hypoxia-responsive transcriptional enhancer. Proc Natl Acad Sci USA 90: 3928-3932, 1993.
43. Payen VL, Brisson L, Dewhirst MW and Sonveaux P: Common responses of tumors and wounds to hypoxia. Cancer J 21: 75-87, 2015.
44. Kitowska A and Pawełczyk T: N-myc downstream regulated 1 gene and its place in the cellular machinery. Acta Biochim Pol 57: 15-21, 2010.
45. Wang Q, Li LH, Gao GD, Wang G, Qu L, Li JG and Wang CM: HIF-1α up-regulates NDRG1 expression through binding to NDRG1 promoter, leading to proliferation of lung cancer A549 cells. Mol Biol Rep 40: 3723-3729, 2013.
46. Salnikow K, Davidson T, Zhang Q, Chen LC, Su W and Costa M: The involvement of hypoxia-inducible transcription factor-1-dependent pathway in nickel carcinogenesis. Cancer Res 63: 3524-3530, 2003.
47. Said HM, Hagemann C, Staab A, Stojic J, Kühnel S, Vince GH, Flentje M, Roosen K and Vordermark D: Expression patterns of the hypoxia-related genes osteopontin, CA9, erythropoietin, VEGF and HIF-1alpha in human glioma in vitro and in vivo. Radiother Oncol 83: 398-405, 2007.
48. Blaes J, Weiler M, Sahm F, Hentschel B, Osswald M, Czabanka M, Thomé CM, Schliesser MG, Pusch S, Luger S, *et al*: NDRG1 prognosticates the natural course of disease in WHO grade II glioma. J Neurooncol 117: 25-32, 2014.
49. Wang Z, Wang F, Wang WQ, Gao Q, Wei WL, Yang Y and Wang GY: Correlation of N-myc downstream-regulated gene 1 overexpression with progressive growth of colorectal neoplasm. World J Gastroenterol 10: 550-554, 2004.
50. Guan RJ, Ford HL, Fu Y, Li Y, Shaw LM and Pardee AB: *Drg-1* as a differentiation-related, putative metastatic suppressor gene in human colon cancer. Cancer Res 60: 749-755, 2000.
51. Lai LC, Su YY, Chen KC, Tsai MH, Sher YP, Lu TP, Lee CY and Chuang EY: Down-regulation of *NDRG1* promotes migration of cancer cells during reoxygenation. PLoS One 6: e24375, 2011.
52. Luo EC, Chang YC, Sher YP, Huang WY, Chuang LL, Chiu YC, Tsai MH, Chuang EY and Lai LC: MicroRNA-769-3p down-regulates NDRG1 and enhances apoptosis in MCF-7 cells during reoxygenation. Sci Rep 4: 5908, 2014.
53. Banz VM, Medová M, Keogh A, Furer C, Zimmer Y, Candinas D and Stroka D: Hsp90 transcriptionally and post-translationally regulates the expression of NDRG1 and maintains the stability of its modifying kinase GSK3beta. Biochim Biophys Acta 1793: 1597-1603, 2009.
54. Dominick G, Bowman J, Li X, Miller RA and Garcia GG: mTOR regulates the expression of DNA damage response enzymes in long-lived Snell dwarf, GHRKO, and PAPPA-KO mice. Aging Cell 16: 52-60, 2017.
55. Khaliullina H, Love NK and Harris WA: Nutrient-deprived retinal progenitors proliferate in response to hypoxia: Interaction of the HIF-1 and mTOR pathway. J Dev Biol 4: 17, 2016.
56. Berta MA, Mazure N, Hattab M, Pouysségur J and Brahimi-Horn MC: SUMOylation of hypoxia-inducible factor-1alpha reduces its transcriptional activity. Biochem Biophys Res Commun 360: 646-652, 2007.

57. Cheng J, Kang X, Zhang S and Yeh ET: SUMO-specific protease 1 is essential for stabilization of HIF1alpha during hypoxia. Cell 131: 584-595, 2007.
58. Gosslar U, Schmid RM and Holzmann B: Regulation of Egr-1-dependent gene expression by the C-terminal activation domain. Biochem Biophys Res Commun 255: 208-215, 1999.
59. Huang RP, Fan Y, Ni Z, Mercola D and Adamson ED: Reciprocal modulation between Sp1 and Egr-1. J Cell Biochem 66: 489-499, 1997.
60. Koizume S, Ito S, Nakamura Y, Yoshihara M, Furuya M, Yamada R, Miyagi E, Hirahara F, Takano Y and Miyagi Y: Lipid starvation and hypoxia synergistically activate *ICAM1* and multiple genes in an Sp1-dependent manner to promote the growth of ovarian cancer. Mol Cancer 14: 77, 2015.
61. Turkoglu SA and Kockar F: SP1 and USF differentially regulate ADAMTS1 gene expression under normoxic and hypoxic conditions in hepatoma cells. Gene 575: 48-57, 2016.
62. Zhang P, Tchou-Wong KM and Costa M: Egr-1 mediates hypoxia-inducible transcription of the *NDRG1* gene through an overlapping Egr-1/Sp1 binding site in the promoter. Cancer Res 67: 9125-9133, 2007.
63. Salnikow K, Kluz T, Costa M, Piquemal D, Demidenko ZN, Xie K and Blagosklonny MV: The regulation of hypoxic genes by calcium involves c-Jun/AP-1, which cooperates with hypoxia-inducible factor 1 in response to hypoxia. Mol Cell Biol 22: 1734-1741, 2002.
64. Lee M, Song SU, Ryu JK and Suh JK: Sp1-dependent regulation of the tissue inhibitor of metalloproteinases-1 promoter. J Cell Biochem 91: 1260-1268, 2004.
65. Miki N, Ikuta M and Matsui T: Hypoxia-induced activation of the retinoic acid receptor-related orphan receptor alpha4 gene by an interaction between hypoxia-inducible factor-1 and Sp1. J Biol Chem 279: 15025-15031, 2004.
66. Archer MC: Role of sp transcription factors in the regulation of cancer cell metabolism. Genes Cancer 2: 712-719, 2011.
67. Koshikawa N, Hayashi J, Nakagawara A and Takenaga K: Reactive oxygen species-generating mitochondrial DNA mutation up-regulates hypoxia-inducible factor-1alpha gene transcription via phosphatidylinositol 3-kinase-Akt/protein kinase C/histone deacetylase pathway. J Biol Chem 284: 33185-33194, 2009.
68. Seifeddine R, Fulchignoni-Lataud MC and Massaad-Massade L: Down-regulation of C/EBPalpha in breast cancer cells by hypoxia-estrogen combination is mainly due to hypoxia. Anticancer Res 29: 1227-1231, 2009.
69. Seifeddine R, Dreiem A, Blanc E, Fulchignoni-Lataud MC, Le Frère Belda MA, Lecuru F, Mayi TH, Mazure N, Favaudon V, Massaad C, *et al*: Hypoxia down-regulates CCAAT/enhancer binding protein-alpha expression in breast cancer cells. Cancer Res 68: 2158-2165, 2008.
70. Gery S, Tanosaki S, Bose S, Bose N, Vadgama J and Koeffler HP: Down-regulation and growth inhibitory role of C/EBPalpha in breast cancer. Clin Cancer Res 11: 3184-3190, 2005.
71. Chen Z, Zhang D, Yue F, Zheng M, Kovacevic Z and Richardson DR: The iron chelators Dp44mT and DFO inhibit TGF-β-induced epithelial-mesenchymal transition via up-regulation of N-Myc downstream-regulated gene 1 (NDRG1). J Biol Chem 287: 17016-17028, 2012.
72. Hayashi M, Martinez OM, Krams SM, Burns W and Esquivel CO: Cytokines are involved in the rejection of small intestine allografts. Transplant Proc 29: 1802, 1997.
73. Dooley S, Said HM, Gressner AM, Floege J, En-Nia A and Mertens PR: Y-box protein-1 is the crucial mediator of antifibrotic interferon-gamma effects. J Biol Chem 281: 1784-1795, 2006.