

# Mitogen-inducible gene 6 (MIG-6), adipophilin and tuftelin are inducible by hypoxia

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**Abstract** Adaptation to hypoxia is essential for tumor progression. Transcriptional activation of hypoxia-regulated genes is mediated by hypoxia-inducible factor 1 (HIF-1), a heterodimer of HIF-1 $\alpha$  and ARNT (Ah receptor nuclear translocator; HIF-1 $\beta$ ). Using representational difference analysis, we identified three novel hypoxia-inducible genes: MIG-6 (gene 33), adipophilin and tuftelin. The mRNAs for these genes were inducible by 1% O<sub>2</sub> in the human HepG2 and MCF-7 cell lines. Hypoxic induction of the MIG-6 and tuftelin proteins was also observed. Induction was ARNT-dependent. Induction also occurred in livers of mice treated with CoCl<sub>2</sub>, which mimics hypoxia. The potential roles of these genes in adaptation to hypoxia and in tumorigenesis will be of considerable interest.

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**Key words:** Hypoxia; MIG-6; Adipophilin; Tuftelin; DEC1; ARNT

## 1. Introduction

Mammalian cells respond to low oxygen levels in part by eliciting adaptive changes in gene expression. This complex response involves numerous genes with diverse biological functions. In general, these adaptive responses help to establish oxygen homeostasis in oxygen-deficient tissues by increasing O<sub>2</sub> delivery (erythropoiesis, and angiogenesis) or by providing metabolic adaptation to reduced O<sub>2</sub> availability (glycolysis). Transcriptional activation of most hypoxia-responsive genes is mediated by hypoxia-inducible factor 1

(HIF-1), which is a heterodimer of two basic helix–loop–helix (bHLH)–Per–Arnt–Sim (PAS) proteins, HIF-1 $\alpha$  and ARNT (aryl hydrocarbon receptor nuclear translocator; also termed HIF-1 $\beta$ ) [1]. ARNT also heterodimerizes with several other bHLH–PAS proteins. For example, it dimerizes with the Ah receptor (AHR), thereby regulating the transcription of a number of genes involved in metabolism of carcinogens and other xenobiotics [2]. The expression of ARNT protein is maintained at constant levels in normoxic and hypoxic cells.

In contrast to ARNT, the HIF-1 $\alpha$  subunit is unique to HIF-1, and its expression and activity are tightly regulated by cellular O<sub>2</sub> concentration [3]. HIF-1 $\alpha$  regulation occurs at multiple levels including mRNA expression, protein stability, nuclear localization and transactivation. However, the regulation of the HIF-1 $\alpha$  stability via the ubiquitin–proteasome pathway appears to be the most important regulatory mechanism. In normoxia, oxygen-dependent prolyl hydroxylases catalyze modifications of HIF-1 $\alpha$ , that allow the von Hippel–Lindau tumor suppressor protein to bind HIF-1 $\alpha$  and target it for ubiquitination and proteasomal degradation, whereas under hypoxic conditions this degradation is blocked [4; reviewed in [3]]. In addition, abrogation of asparagine hydroxylation of the C-terminal HIF-1 $\alpha$  transactivation domain has been shown to be required for the interaction of HIF-1 $\alpha$  with the co-activator p300 and is therefore a prerequisite for full induction of HIF-1 $\alpha$  [5].

In human cancers, adaptation to hypoxia appears to be a critical step in tumor progression to a more malignant stage. After tumors reach the size of several millimeters in diameter, diffusion is not sufficient to supply oxygen into the center of the tumor, thus resulting in a hypoxic condition. Overexpression of HIF-1 $\alpha$  has been detected in tumor cells where it may mediate adaptive responses to hypoxia, critical to tumor progression. For example, hypoxia induces vascular endothelial growth factor in tumors, thus enabling them to establish a vascular supply that allows them to grow beyond the volume at which O<sub>2</sub> becomes diffusion-limited. Another essential adaptation of tumor cells to hypoxia is an increased rate of glycolysis by induction of glycolytic enzymes, which allows energy production in low oxygen conditions. In addition to intratumoral hypoxia, genetic alterations in tumor suppressor genes and oncogenes has been shown to result in overexpression of HIF-1 $\alpha$  in tumors [3]. Obviously, genes mediating adaptive responses to hypoxia play important roles in tumor development. With this in mind, we set out to look for novel hypoxia-inducible genes in human cell lines using representational difference analysis (RDA).

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**Abbreviations:** AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; bHLH, basic helix–loop–helix; ChoB, Chinese hamster ovary B; DEC1, differentiated embryo chondrocyte 1; HIF-1, hypoxia-inducible factor 1; MIG-6, mitogen-inducible gene 6; PAS, Per–Arnt–Sim; PCR, polymerase chain reaction; RDA, representational difference analysis

## 2. Materials and methods

### 2.1. Cells and cell culture

HepG2 (human hepatocellular carcinoma) and MCF-7 (human mammary adenocarcinoma) cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). A mutant strain of the mouse hepatoma cell line Hepa1c1c7 (Hepa-1) deficient in ARNT (c4) was previously constructed in this laboratory [6]. All of the cell lines were cultured in nucleoside-free  $\alpha$ -minimal essential medium (Life Technologies, Rockville, MD, USA) supplemented with 10% fetal calf serum (Omega, Tarzana, CA, USA), 100 U/ml of penicillin and streptomycin (Gemini Bio-Products, Woodland, CA, USA), and 0.25 U/ml of amphotericin (Omega). The hypoxic treatment was performed in a sealed incubation chamber (Billups-Rothenburg, Del Mar, CA, USA) flushed with a gas mixture containing 1% oxygen, 5% carbon dioxide and 94% nitrogen. The cells were exposed to hypoxia at 90% confluency. Fresh medium was exchanged immediately prior to hypoxia exposure since many hypoxia-inducible genes are also induced by hypoglycemia [7].

### 2.2. RDA

HepG2 and MCF-7 cells were exposed to either normoxia or hypoxia (1% oxygen) for 16 h. Messenger RNA was isolated using the Fast Track 2.0 mRNA isolation kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Double-stranded cDNA populations were synthesized from mRNA using standard techniques. RDA was performed as described previously [8]. Briefly, amplicons representing the hypoxia-treated (tester) and control (driver) cDNA fragments were first created from the cDNA populations.

To accomplish this, the cDNAs were digested with the restriction enzyme Dpn II. Identical adaptors were then placed on both ends of both cDNA populations and the adaptor-ligated cDNA fragments were amplified using additional unliganded adaptors as polymerase chain reaction (PCR) primers to create amplicons. All adaptors were then removed from both amplicon species and new adaptors were ligated onto only the tester amplicons. To enrich the tester amplicons with upregulated gene fragments, subtractive cross-hybridization of tester and driver amplicons was performed. During this phase, a great excess of adaptor-less driver amplicons was hybridized to tester amplicons, followed by PCR amplification of the hybridization mixture using adaptors specific for the tester amplicons as primers. The resultant difference product was then cleaved again with Dpn II and a new adaptor sequence placed at the ends. The original adaptor-less driver amplicons were again used with this new 'tester' species in the next subtractive hybridization/PCR reaction. This cycle was then repeated three additional times with fresh adaptors placed on the resultant difference product each time. After four cycles of subtractive hybridization/PCR amplification, the difference product was cloned into the pTarget vector (Promega, Madison, WI, USA) using the T/A overhang technique. Clones were confirmed for induction by hypoxia using a modified reverse Northern technique whereby the inserts were immobilized on duplicate nitrocellulose membranes and probed with normalized [ $^{32}$ P]dATP-labeled (Prime-A-Gene Labeling kit, Promega) amplicons from hypoxia-treated or untreated cells. The induction of upregulated clones was confirmed by Northern analysis and they were then sequenced using an Applied Biosystems PRISM cycle sequencing system (Laragen, Santa Monica, CA, USA). Identification of the isolated cDNA fragments was established by BLAST searches of the NCBI Databank.

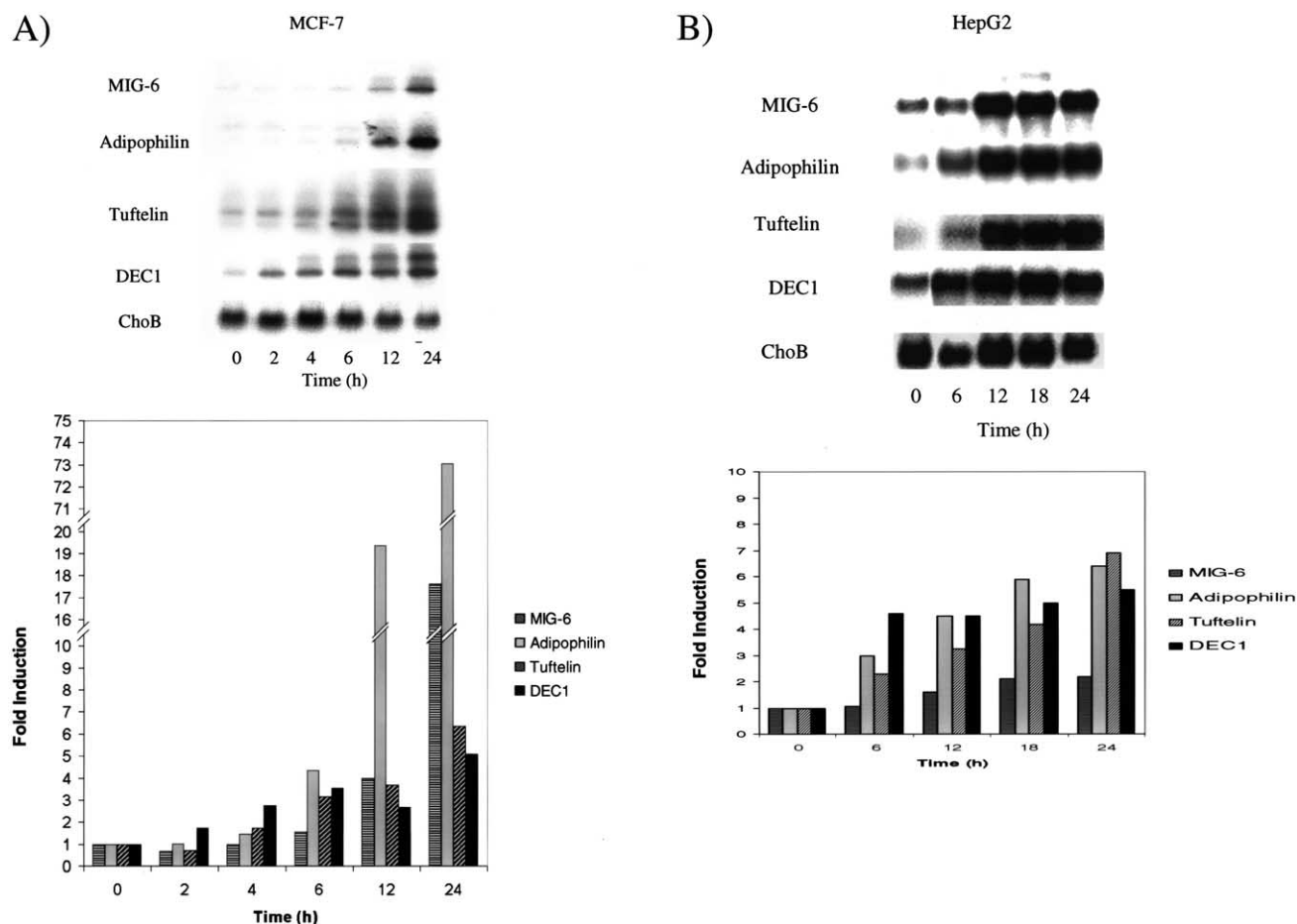


Fig. 1. Hypoxic induction of mRNAs for the cloned genes in MCF-7 (Fig. 1A) and HepG2 (Fig. 1B) cell lines. The cells were exposed to 1% O<sub>2</sub> for the times indicated. Constitutively expressed ChoB was used to correct for inter-lane variability. The results of phosphor-imager analysis of band intensities are presented. (For the MCF-1 cell line, data for the longer transcript were used for tuftelin and data for the shorter transcript for DEC1.)

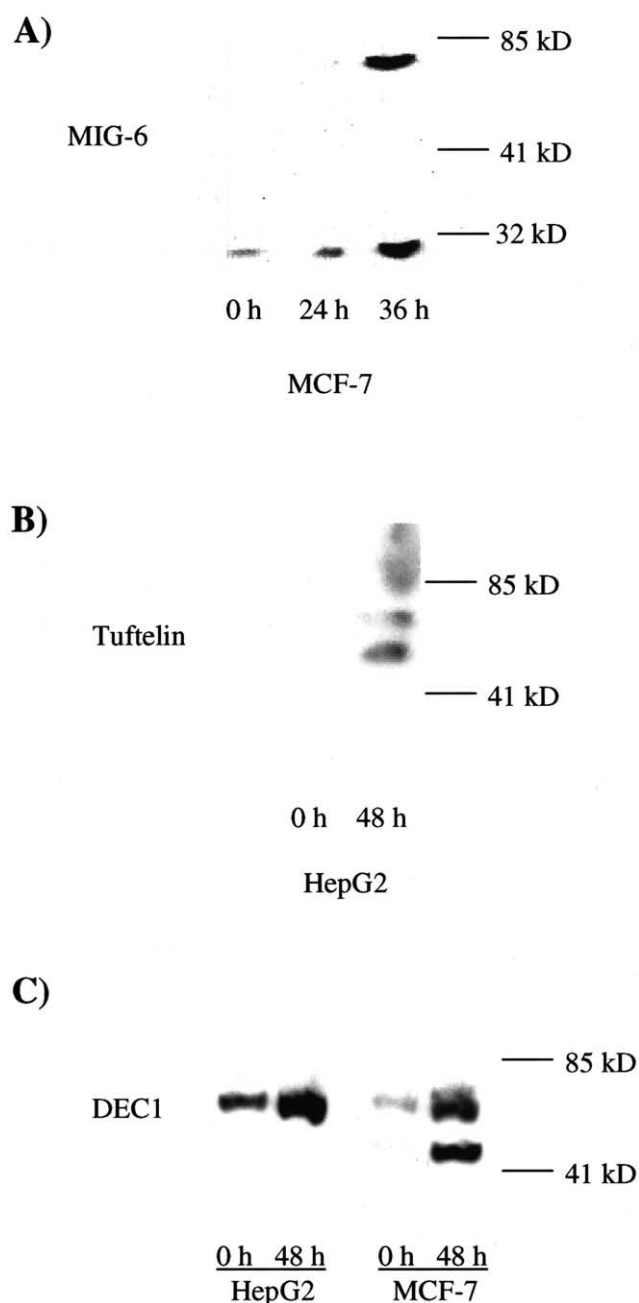


Fig. 2. Hypoxic induction of MIG-6 (A), tuftelin (B) and DEC1 (C) proteins in MCF-7 and HepG2 cells. Cells were exposed to 1% O<sub>2</sub> for the times indicated.

### 2.3. Northern analysis

Cells were exposed to normoxia or hypoxia for the periods indicated. mRNA was isolated using the Fast Track 2.0 mRNA isolation kit according to the manufacturer's instructions (Invitrogen). Northern blotting was performed according to standard protocols. Labeling of the probes was done by random primed [<sup>32</sup>P]dATP incorporation (Prime-A-Gene Labeling kit, Promega). Phosphor-imaging analysis was performed on a Molecular Dynamics 455SI phosphorimager with correction for inter-lane load variability by comparison with ChoB (Chinese hamster ovary B), a constitutively expressed gene that is not responsive to hypoxia. ChoB cDNA was kindly provided by Dr. Harvey Herschman.

### 2.4. Western analysis

Cells were exposed to normoxia or hypoxia for 48 h or for the

periods indicated. At harvest, cells were scraped, resuspended in phosphate-buffered saline and pelleted by centrifugation at 5 × g for 5 min at 4°C. The pellets were then suspended in SDS-PAGE sample buffer (10% sodium dodecyl sulfate (SDS), 0.5 M Tris-HCl pH 6.8, 2.5 mM ethylenediamine tetraacetic acid (EDTA), 47% glycerol, 0.5% bromophenol blue, non-reducing), vortexed and sonicated for three 1 s intervals. 24 µg of each sample was reduced by boiling in the presence of 0.1% β-mercaptoethanol and separated by SDS-PAGE in 10% polyacrylamide gels. Proteins were immobilized onto Immobilon-P Western membrane (Millipore, Bedford, MA, USA), and probed with primary antibodies to DEC1 (differentiated embryo chondrocyte 1; Chemi-Con International, Temecula, CA, USA), tuftelin [9] and MIG-6 (mitogen-inducible gene 6, kindly provided by Dr. A. Makkinje). Membranes were then incubated with secondary antibody consisting of either goat anti-mouse IgG (MIG-6) or goat anti-rabbit IgG (tuftelin, DEC1) conjugated to horseradish peroxidase in blotto at 1:2000 dilution for 1 h at room temperature. Proteins were detected with Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) on Kodak BioMax MR film.

### 2.5. CoCl<sub>2</sub> treatment of mice

Treatment of the mice with CoCl<sub>2</sub>, a chemical known to mimic physiological hypoxia in animals [10], was performed by intraperitoneal injection of C57BL/6 mice with 75 mg/kg of CoCl<sub>2</sub> or vehicle (saline). Mice were killed 10 h later and organs harvested and stored in liquid nitrogen.

## 3. Results

### 3.1. Identification of clones representing hypoxia-inducible genes

RDA experiments performed on the human hepatocellular carcinoma (HepG2) or the human mammary adenocarcinoma (MCF-7) cell lines comparing cDNAs from untreated cells and from cells treated for 16 h with 1% O<sub>2</sub>, lead to the isolation of several clones that were confirmed to be hypoxia-inducible by reverse Northern analysis. As expected, many of these clones represented previously identified hypoxia-inducible genes, including heme-oxygenase 1, enolase 1, lactate dehydrogenase A, plasminogen activator inhibitor 1, alpha-1-antitrypsin and hexokinase 2. From the RDA experiment with MCF-7 cells, hypoxia-inducible clones representing cDNAs for tuftelin and DEC1 (differentiated embryo chondrocyte 1; also known as Stra13 and Sharp-2) were also isolated (Fig. 1A). During the course of this work DEC1 was identified by other investigators as well as ourselves as being hypoxia-inducible [11–13]. From the HepG2 cell line we identified two genes, MIG-6 (also called gene 33) and adipophilin,

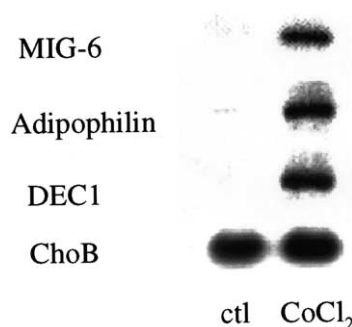


Fig. 3. Induction of MIG-6, adipophilin and DEC1 mRNAs in livers of mice treated with intraperitoneal injection of 75 mg/kg of CoCl<sub>2</sub> or vehicle (saline) for 10 h. ChoB mRNA was used to control for loading.

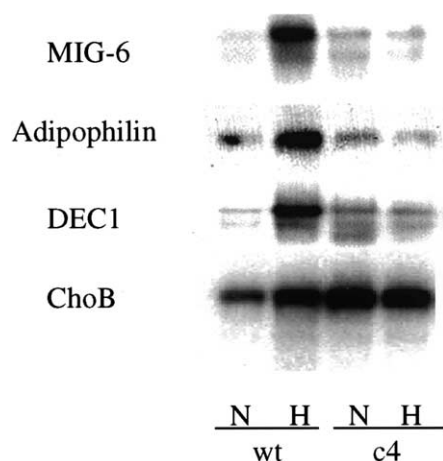


Fig. 4. Hypoxic induction of MIG-6, adipophilin and DEC1 mRNAs in wild-type (wt) Hepa-1 cell line and in a mutant Hepa-1 cell line deficient in expression of ARNT (c4) after 16 h exposure to 1% O<sub>2</sub>. ChoB was used as a load control.

that showed strong hypoxic responses and that were previously not known to be hypoxia-inducible (Fig. 1B).

### 3.2. Characterization of hypoxic induction

MIG-6, adipophilin, tuftelin and DEC1 were responsive to hypoxia in both MCF-7 and HepG2 cell lines (Fig. 1). Time-course experiments revealed a time-dependent upregulation of mRNA expression of all of the genes in both the HepG2 and MCF-7 cell lines, with maximal inductions at the longest time point studied, i.e. 24 h (Fig. 1). The maximal inductions of tuftelin and of DEC1 mRNAs were relatively similar in both cell lines, ranging in different experiments from 6.5-fold to nine-fold for tuftelin and from four- to six-fold for DEC1. MIG-6 showed more inter-experimental variation and the induction appeared to be stronger in MCF-7 cells compared to HepG2 cells (maximal fold inductions were seven- to 17-fold and two- to 12-fold, respectively). Adipophilin showed six- to seven-fold induction in HepG2, and the induction was more marked and variable in MCF-7 cell line (10- to 70-fold). The variations in inducibility, particularly for MIG-6 and adipophilin mRNAs in MCF-7 cells, are probably due to the difficulties in accurately detecting the very low levels of these mRNAs in normoxic cells.

Western blotting analyses showed that MIG-6, tuftelin and DEC1 were inducible also at the protein level (Fig. 2). (We could not detect the adipophilin protein with available antibodies.)

DEC1, MIG-6 and adipophilin mRNAs were also observed to be inducible in livers of mice treated with CoCl<sub>2</sub>, a chemical known to mimic physiological hypoxia in animals [10] (Fig. 3). MIG-6, adipophilin and DEC1 were also inducible by hypoxia in the mouse hepatoma cell line, Hepa-1, but did not respond to hypoxia in an ARNT-deficient derivative of Hepa-1 cells (Fig. 4). This demonstrates that the hypoxic induction of these genes is dependent on ARNT and therefore on HIF-1.

## 4. Discussion

In the present study we identified three novel hypoxia-responsive genes: MIG-6, adipophilin and tuftelin. A fourth

gene, DEC1, was also identified; its hypoxic induction has recently been published by us [11] and others [12,13]. The genes identified encode a spectrum of proteins with diverse cellular functions. MIG-6 mRNA levels have been found to increase sharply in response to various mitogens and commonly occurring chronic stress stimuli [14,15]. The rapid stress-induced expression of MIG-6 suggests that it is an immediate early gene that may serve to reprogram cells in order to enable them to respond to persistent stress. Indeed, MIG-6 has recently been shown to activate stress-activated protein kinases (SAPKs). SAPKs in turn stimulate MIG-6 transcription and this positive feedback cycle thus maintains chronic activation of SAPK-dependent mechanisms [14]. MIG-6 contains several SH2 domains, which indicate that it may be an adaptor protein that binds monomeric GTPases. Our results indicate that in addition to acting as a mediator for previously known cell stressors, MIG-6 may also act as an adaptor molecule in mediating signal transduction in the cellular responses to hypoxia. Given its widespread tissue distribution, MIG-6 may have a relatively universal function in the body [16].

Adipophilin is expressed at the surface of lipid droplets in many different cell types and tissues that accumulate lipids, such as lactating mammary epithelial cells, adrenal cortex cells and hepatocytes [17]. Adipophilin may thus have a general role in deposition and turnover of lipids. Adipophilin has recently been found to be upregulated in renal cell carcinoma [18]. Interestingly, it has been shown that activation of AhR, another dimerization partner for ARNT in addition to HIF-1 $\alpha$ , inhibits adipose differentiation [19]. AHR ligands (e.g. dioxin) and hypoxia may thus exert opposite effects on adipocyte differentiation via competition for the common factor, ARNT.

Tuftelin is known to play an important role during the development and mineralization of enamel in the teeth [20]. Tuftelin is, however, also expressed in many tissues other than teeth, including kidney, lung and testis in the mouse [20,21], suggesting that the protein may have a more general physiological role yet to be determined. Therefore, it is interesting that tuftelin expression is induced by cellular oxygen deprivation.

We show here that induction of DEC1 is dependent on ARNT, in addition to HIF-1 $\alpha$  [12]. DEC1 belongs to the bHLH family of transcription factors that play important roles in the regulation of cell proliferation, differentiation, and apoptosis. The protein has been shown to repress activated transcription via interactions with the general factors of the basal transcription machinery [22]. DEC1 has been observed to be upregulated in a large variety of common tumors [13]. As a transcriptional regulator involved in differentiation and cell cycle regulation, DEC1 may play a critical role in carcinogenesis.

In conclusion, we report here identification of three novel hypoxia-inducible genes: MIG-6 (gene 33), adipophilin and tuftelin. Our experiments show that the hypoxic inductions are dependent on ARNT (HIF-1) and occur also in vivo in mice under hypoxia-mimicking conditions. Low oxygen levels elicit a broad spectrum of cellular responses that are crucial for physiologic homeostasis and may be involved in pathological conditions. Therefore, searching for the potential roles of these novel hypoxia-inducible genes in physiological adaptation to hypoxia and in pathways involved in tumorigenesis will be of considerable interest.

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## References

- [1] Semenza, G.L. (2000) *Biochem. Pharmacol.* 59, 47–53.
- [2] Hankinson, O. (1995) *Annu. Rev. Pharmacol. Toxicol.* 35, 307–340.
- [3] Semenza, G. (2002) *Trends Mol. Med.* 8, 62–67.
- [4] Mahon, P.C., Hirota, K. and Semenza, G.L. (2001) *Genes Dev.* 15, 2675–2686.
- [5] Lando, D., Peet, D.J., Whelan, D.A., Gorman, J.J. and White-law, M.L. (2002) *Science* 295, 858–861.
- [6] Hoffman, E.C., Reyes, H., Chu, F.F., Sander, F., Conley, L.H., Brooks, B.A. and Hankinson, O. (1991) *Science* 252, 954–958.
- [7] Carmeliet, P., Dor, Y., Herbert, J.M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P., Koch, C.J., Ratcliffe, P., Moons, L., Jain, R.K., Collen, D. and Keshert, E. (1998) *Nature* 394, 485–490.
- [8] Chang, D. and Denny, C.T. (1998) in: *Gene Cloning and Analysis by RT-PCR* (Seibert, P.D. and Larrick, J.W., Eds.), pp. 193–202, Eaton Publ., Natick, MA.
- [9] Diekwisch, T.G., Ware, J., Fincham, A.G. and Zeichner-David, M. (1997) *J. Histochem. Cytochem.* 45, 859–866.
- [10] Beru, N., McDonald, J., Lacombe, C. and Goldwasser, E. (1986) *Mol. Cell Biol.* 6, 2571–2575.
- [11] Yoon, D.Y., Buchler, P., Saarikoski, S.T., Hines, O.J., Reber, H.A. and Hankinson, O. (2001) *Biochem. Biophys. Res. Commun.* 288, 882–886.
- [12] Wykoff, C.C., Pugh, C.W., Maxwell, P.H., Harris, A.L. and Ratcliffe, P.J. (2000) *Oncogene* 19, 6297–6305.
- [13] Ivanova, A.V., Ivanov, S.V., Danilkovitch-Miagkova, A. and Lerman, M.I. (2001) *J. Biol. Chem.* 27, 15306–15315.
- [14] Makkinje, A., Quinn, D.A., Chen, A., Cadilla, C.L., Force, T., Bonventre, J.V. and Kyriakis, J.M. (2000) *J. Biol. Chem.* 275, 17838–17847.
- [15] Wick, M., Burger, C., Funk, M. and Muller, R. (1995) *Exp. Cell Res.* 219, 527–535.
- [16] Van Laar, T., Schouten, T. and Van der Eb, A.J. (2001) *Mol. Carcinog.* 31, 63–67.
- [17] Heid, H.W., Moll, R., Schwetlick, I., Rackwitz, H.R. and Keenan, T.W. (1998) *Cell Tissue Res.* 294, 309–321.
- [18] Rae, F.K., Stephenson, S.A., Nicol, D.L. and Clements, J.A. (2000) *Int. J. Cancer* 88, 726–732.
- [19] Shimba, S., Wada, T. and Tezuka, M. (2001) *J. Cell Sci.* 114, 2809–2817.
- [20] Mao, Z., Shay, B., Hekmati, M., Fermon, E., Taylor, A., Dafni, L., Heikinheimo, K., Lustmann, J., Fisher, L.W., Young, M.F. and Deutsch, D. (2001) *Gene* 279, 181–196.
- [21] MacDougall, M., Simmons, D., Dodds, A., Knight, C., Luan, X., Zeichner-David, M., Zhang, C., Ryu, O.H., Qian, Q., Simmer, J.P. and Hu, C.C. (1998) *J. Dent. Res.* 77, 1970–1978.
- [22] Sun, H. and Taneja, R. (2000) *Proc. Natl. Acad. Sci. USA* 97, 4058–4063.