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Hypoxia increases cytoplasmic expression of NDRG1, but is insufficient for its membrane localization in human hepatocellular carcinoma

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Abstract NDRG1 is a hypoxia-inducible protein, whose modulated expression is associated with the progression of human cancers. Here, we reveal that NDRG1 is markedly upregulated in the cytoplasm and on the membrane in human hepatocellular carcinoma (HCC). We demonstrate further that hypoxic stress increases the cytoplasmic expression of NDRG1 in vitro, but does not result in its localization on the plasma membrane. However, grown within an HCC-xenograft in vivo, cells express NDRG1 in the cytoplasm and on the plasma membrane. In conclusion, hypoxia is a potent inducer of NDRG1 in HCCs, albeit requiring additional stimuli within the tumour microenvironment for its recruitment to the membrane.

Keywords: N-myc downstream-regulated gene 1; Hypoxia; HIF-1; Carcinoma, hepatocellular

1. Introduction

The N-myc downstream-regulated gene-1 (NDRG1) is a novel protein initially described as a stress-responsive protein and as being involved in cellular differentiation events [1–3]. Current studies indicate that its putative biological function may be as a tumour suppressor protein, and more specifically as an inhibitor of tumour metastasis [3–8]. Paradoxically, findings relating to the expression of NDRG1 are not always consistent with this tenet. NDRG1 is commonly found to be expressed at a higher level in cancerous than non-cancerous tissue of the same origin, suggesting that the modulated expression of NDRG1 may be tissue- or cell-type specific [9].

NDRG1 is a physiological substrate for serum- and glucocorticoid-induced kinase 1 (SGK1) and glycogen synthase kinase 3 (GSK3) [20]. It is phosphorylated on several residues within three 10-amino acid tandem repeats located near its C-terminus. The expression of NDRG1 can be altered by various physiological conditions and external stimuli, in particular, hypoxia is a key stimulus for its increased expression [10,11]. Central to the regulation of hypoxia-inducible genes is the heterodimeric transcription factor, hypoxia-inducible factor (HIF)-1 [12]. HIF-1 is negatively-regulated at a normal oxygen concentrations, whereas under hypoxic conditions, its alpha-subunit is stabilized and is translocated to the nucleus, where it binds to its β-subunit and activates the transcription of its target genes [13–15].

Hypoxia is a common characteristic and a key stimulus in the pathophysiology of many solid tumours including the highly-aggressive, chemoresistant hepatocellular carcinomas (HCCs) [16–18]. In the present study, we investigated the influence of hypoxia on the expression and subcellular localization of NDRG1 and phosphorylated-NDRG1 in HCC.

2. Materials and methods

2.1. Tissue samples

Resected tissues were collected from patients at the Inselspital in Bern, Switzerland in accordance with standard procedures which were approved by an Institutional Review Board (approval no.: 1.05.01.30.–17) and with the patient's written consent. HCC samples were graded 1–3 according to Edmondson and Steiner and according to the classifications of the World Health Organization ($n = 11$). Hepatic tissue taken from tumour-free resection margins served as normal controls ($n = 6$).

2.2. Antibodies, immuno-histochemistry and -fluorescence

Antibodies were obtained from the following companies: NDRG1 and phosphorylated-NDRG1 (p3xThr 346, 356 and 366) (Kinasource Limited, Scotland, UK), Sp1 and IκBα (both from Santa Cruz, USA), E-cadherin (BD Biosciences), β-actin (Sigma) and HIF-1α (Novus Biologicals). Formalin fixed tissues sections (5-μm-thick) were pressure cooked for 15 min in ChemMate™ Target Retrieval Solution (Dako-Cytomation). Endogenous peroxidase was blocked with 3% H_2O_2 for 10 min and blocked with 1% normal rabbit serum (NRS). Sections were incubated overnight at 4 °C with the primary antibody and immunoreactivity was detected using ABC kits (Vectastain) and 3,3′-diaminobenzidine as the peroxidase substrate or a FITC-conjugated secondary antibody was used for fluorescence microscopy. Cells were grown on glass cover slips, fixed in 4% PBS-buffered formaldehyde,

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Abbreviations: NDRG1, N-myc downstream regulated gene-1; HIF-1, hypoxia-inducible factor-1; HCC, hepatocellular carcinoma; O_2, oxygen; siRNA, short-interfering RNA; DMOG, dimethyloxallyl glycine; GSK3, glycogen synthase kinase 3; SGK-1, serum- and glucocorticoid-induced kinase 1

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permeabilized in methanol for 1 min at −20 °C. Samples were examined with a Leica DMRB microscope and documented using IM50 Leica imaging software.

2.3. Cells and culture conditions

The human HCC cell line, Hep3B, was purchased from the European Collection of Cell Cultures and cultured according to suggested guidelines. Cells were incubated under either normoxic conditions (21% O_2), using a standard CO_2 incubator, or hypoxic conditions (1.5% O_2), using a hypoxia workstation (Ruskinn Technology Limited, West Yorkshire, UK). For a non-hypoxic stabilization of HIF-1α, the cells were incubated in 125 μM dimethyloxallyl glycine [(DMOG), Alexis Biochemicals] for 24 h at 21% O_2. RNA oligonucleotides specific for HIF cDNA were designed with the aid of an online tool (www.ambion.com/techlib/misc/silencer_siRNA_template.html). Double-stranded short-interfering RNA (siRNA) was transfected into Hep3B cells using the supplied Transfection Reagent. The efficiency of RNA interference was determined by real-time PCR.

2.4. Protein isolation and Western blot analysis

For the detection of NDRG1, the total protein content of cells and tissues was extracted using a RIPA lysis buffer and 1:100 dilution of a protease inhibitor cocktail (Sigma). For the detection of HIF-1α in cells and tissues, enriched nuclear–protein extracts were prepared as previously described [19]. For cell fractionation, the ProteoExtract Subcellular Proteome Extraction kit (Calbiochem) was used in accordance with the manufacture's instructions. All proteins were quantified using the BioRad Protein Assay (BioRad). Fifty micrograms of protein were separated by SDS–PAGE, transferred to a nitrocellulose membrane and analyzed by Western blotting using standard protocols. Signals were detected by enhanced chemiluminescence (LiteAblot, Euroclone). For patient sample analysis, the average of three independent protein concentration measurements was used for as an accurate loading control, given that the expression of many housekeeping genes are modified in cancerous tissues.

2.5. Real-time RT-PCR

Total RNA was isolated with Trizol (Invitrogen) and 1 μg of total RNA was reverse transcribed (Qiagen). Primers and FAM-labelled probes were obtained from the Assay-on-Demand for human NDRG1 and HIF-1α and quantitative PCR was performed using an ABI 7700 Sequence Detection System (Applied Biosystems). The C_t values for each target genes were standardized against ribosomal RNA (18S). $\Delta\Delta C_t$ values were calculated by subtracting the ΔC_t values of cells under normoxia from the ΔC_t value of cells under hypoxia. Fold increases were calculated using the formula $2^{-\Delta\Delta C_t}$. All reactions were performed in triplicate, and each experiment was conducted on at least three independent occasions.

2.6. Analysis of the NDRG1 sequence

The domain structure of NDRG1 (Accession No. NP_006087) was determined using the Predict Protein database (http://www.predictprotein.org). Subcellular localization of NDRG4 (Accession No.

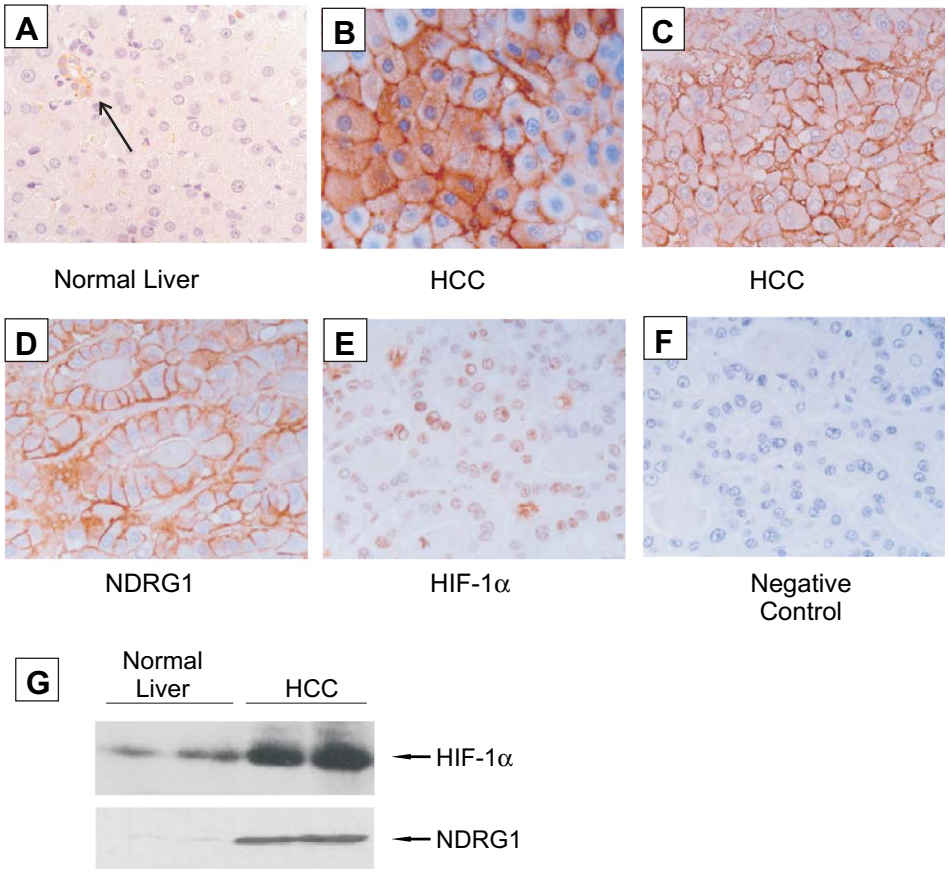

Fig. 1. Expression of NDRG1 and HIF-1α in HCCs by immunohistochemistry. (A–C) NDRG1 expression in (A) normal liver, arrow indicates a weak staining of NDRG1 in biliary cells, (B) cytoplasmic and membrane localization of NDRG1 in a HCC tumour (trabecular type, grade 3) and (C) membrane localization of NDRG1 in a HCC tumour (mixed trabecular and acinar type, grade 2). (D–E) Serial sections of a HCC tumour (mixed trabecular and acinar type, grade 2) for (D) cytoplasmic and membrane staining of NDRG1, (E) nuclear localization of HIF-1α, and (F) a negative control to illustrate the specificity of the reaction (200× magnification). (G) Western blot analysis of HIF-1α and NDRG1 expression in HCC compared to normal liver tissue.

AAH11795) was predicted from pTARGET database (http://bioinformatics.albany.edu/~ptarget/). Sequence homology for NDRG1 and NDRG4 were determined using the SIM alignment tool for proteins (http://www.expasy.org/tools/sim-prot.html).

2.7. Hep3B liver-xenograft model

Experiments were performed according to the National Institute of Health's *Guidelines for the Care and Use of Laboratory Animals* and with the approval of the local Animal Ethics Committee. Liver-tumour xenografts were grown in 4-week-old, male nude mice [Hsd:Athymic Nude-***Foxn1***^{nu} (Harlan Netherlands)] by injecting one million Hep3B into the subcapsular space of the left lateral liver lobe. After 7 weeks, the animals were sacrificed and the tumours were excised for analysis.

3. Results

3.1. Expression and cellular localization of NDRG1 and HIF-1α in human HCC

In normal liver tissue, NDRG1 protein was found to be weakly expressed in the biliary epithelial cells, whereas the hepatocytes were negative (Fig. 1A). In contrast, the cells of HCCs were highly immunoreactive (Fig. 1B and C). Within a single tumour, 50–90% of the tumour cells stained positive for NDRG1 and various expression patterns were observed. NDRG1 expression was found both in the cytoplasm

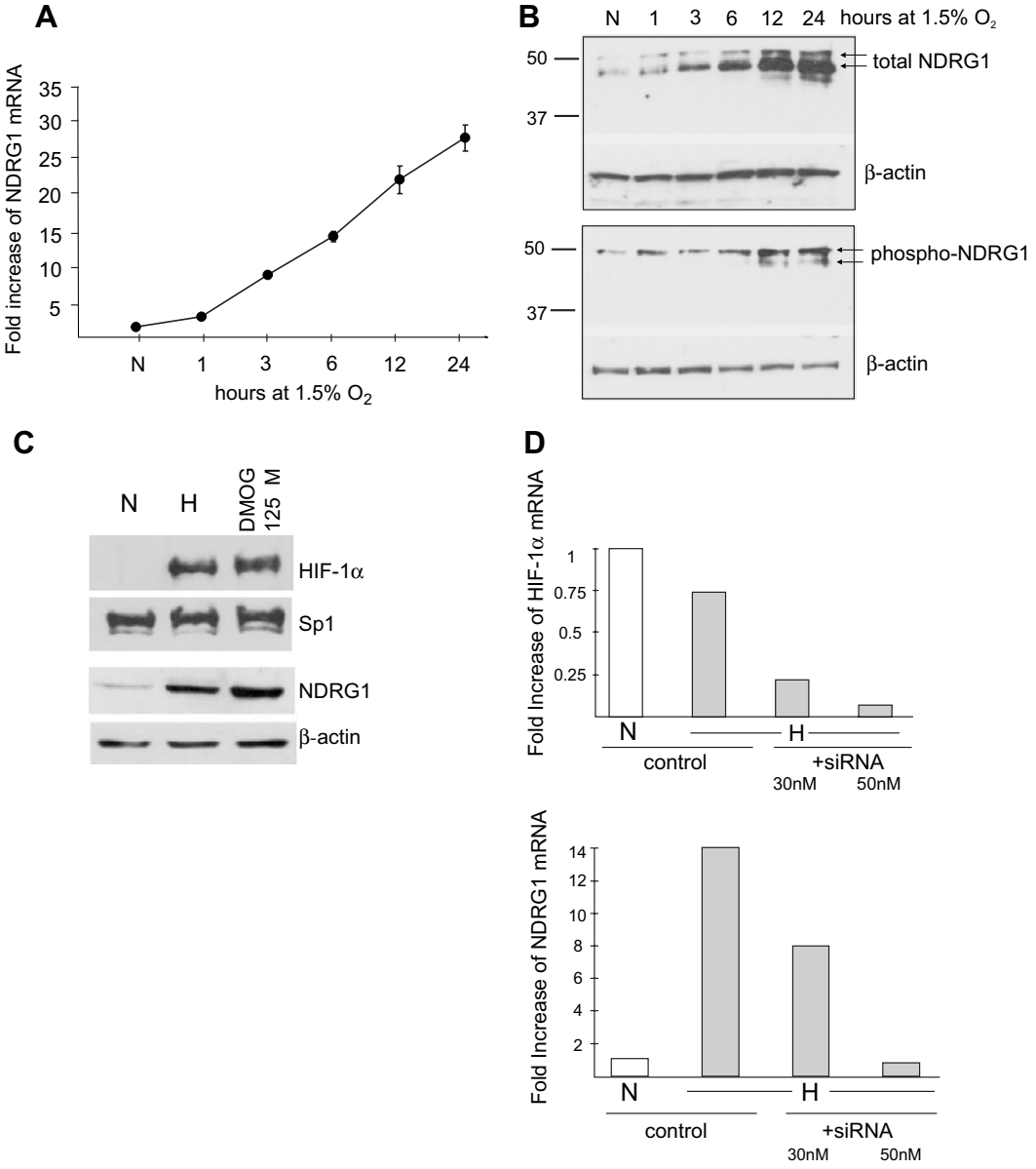

Fig. 2. Hypoxia upregulates the expression of NDRG1 in a HIF-1 dependent manner. (A) Quantitative real-time RT-PCR for NDRG1 in Hep3B cells in response to hypoxia (1.5% O_2). (B) Western blot analysis of NDRG1 in Hep3B cells in response to hypoxia. Upper panel: antibody against total NDRG1 protein and lower panel: antibody against phospho-NDRG1. Arrows indicate upper and lower bands. β-actin was used as a loading control. (C) Western blot analysis for NDRG1 and HIF-1α of cells exposed either to N: 21% O_2, H: 1.5% O_2 or 125 μM DMOG for 24 h. β-Actin (42 kDa) and Sp1 (97 kDa) were used as loading controls. (D) Effect of HIF-1α gene silencing on HIF-1α (upper panel) and NDRG1 (lower panel) mRNA expression. Hep3B cells were transfected with specific HIF-1α siRNA (30 nM and 50 nM) and a control siRNA (50 nM) for 24 h followed by exposure to 1.5% O_2 for 16 h.

(Fig. 1B) and on the membrane (Fig. 1C). Hypoxic stress is a known inducer of NDRG1. By using HIF-1α as a marker for tumour hypoxia, we demonstrated in serial sections of a HCC tumour the concomitant expression of NDRG1 (Fig. 1D) and HIF-1α (Fig. 1E). Furthermore, both HIF-1α and NDRG1 protein levels were substantially higher in HCCs than in normal hepatic tissue (Fig. 1G). These data reveal NDRG1 to be markedly over-expressed in HCCs and to be located within both the cytoplasm and the plasma membrane, and that its expression is co-localized with the stabilization of HIF-1α.

3.2. Hypoxia upregulates the expression of NDRG1 in a HIF-1-dependent manner

To assess the effect of hypoxia on the expression of NDRG1 in HCC cells in vitro, we used as a model the human HCC cell line, Hep3B. Hypoxia (1.5% O_2) induced an increase in the mRNA level of NDRG1 after 1–3 h which increased to 30-fold by 24 h (Fig. 2A). NDRG1 protein was detected as a doublet when analyzed by Western blot using an antibody against its total protein. The more rapidly migrating lower band increased after 3 h of hypoxia, and continued to rise for up to 24 h (Fig. 2B, upper panel). The slower migrating upper band also increased albeit only after 12 h at 1.5% O_2. The decrease in the electrophoretic mobility of the upper band possibly reflects a C-terminal phosphorylation of NDRG1 by GSK3, as recently shown for the cervical cancer cell line-HeLa [20]. To determine the phosphorylation state of NDRG1, the same extracts where tested with an antibody raised against phosphorylated NDRG1 at Thr 346, Thr 356 and Thr 366, which is targeted by serum- and glucocorticoid-induced kinase 1 [20]. Phosphorylated NDRG1 was present in normoxic cells and was detected at a similar molecular weight to that of the upper band of the total protein. Both the upper and lower band increased after 12–24 h at 1.5% O_2 thus demonstrating a hypoxia-induced increase of phosphorylated NDRG1 protein by SGK and GSK3 (Fig. 2B, lower panel).

Hypoxia can upregulate genes in a manner that is either dependent upon or independent of HIF-1 [21]. To ascertain whether the upregulation of NDRG1 in liver cells is a consequence of HIF-1 activation, cells were treated with the non-selective prolyl hydroxylase inhibitor, DMOG. Under normoxia (21% O_2), DMOG stabilized HIF-1α and resulted in the simultaneous upregulation of NDRG1 (Fig. 2C). Furthermore, selective silencing of the HIF-1α gene with specific siRNA abolished the hypoxia-induced expression of NDRG1 mRNA in a dose-dependent manner (Fig. 2D). Taken together, this shows that hypoxia and HIF-1 are strong transcriptional inducers of NDRG1 in HCC cells.

3.3. Domain composition of NDRG1

We next analyzed the sequence of NDRG1 for domains which would implicate it as a membrane-associated protein (Fig. 1C). Within its sequence, we identified a highly hydrophobic 20-amino-acid sequence which represents either a potential transmembrane domain or an anchor to the cytosolic face of the lipid bi-layer (Fig. 3A). Its family member, NDRG4 is classified as a type II membrane protein (pTARGET analysis gives 87.6% confidence of plasma membrane localization). A comparative sequence analysis of NDRG1 with NDRG4 revealed a 61% homology in the transmembrane domain identified in NDRG4 (Fig. 3B).

3.4. Subcellular localization of NDRG1 in response to hypoxia in vitro and in vivo

NDRG1 expression has been described to be translocated within the cell in response to exogenous stimuli [4]. We next examined whether hypoxia induces a relocation of NDRG1, in particular to its predominant localization site in HCC, the plasma membrane. NDRG1 was ubiquitously expressed at a low level in the cytoplasm of Hep3B cells under normoxic conditions (Fig. 4A). After 24 h of hypoxic stress, the cytoplasmic level of the NDRG1 increased, but did not translocate to the plasma membrane (Fig. 4B). These results were confirmed by cell fractionation, NDRG1 was not detected in the membrane fraction of either normoxic or hypoxic Hep3B cells (Fig. 4C). An increased expression of both total and phosphorylated protein was detected in the cytoplasm of hypoxic cells.

To verify that the absence of NDRG1 on the plasma membrane is not attributable to a defect in the trafficking machinery in Hep3B cells, these cells were grown in vivo using an

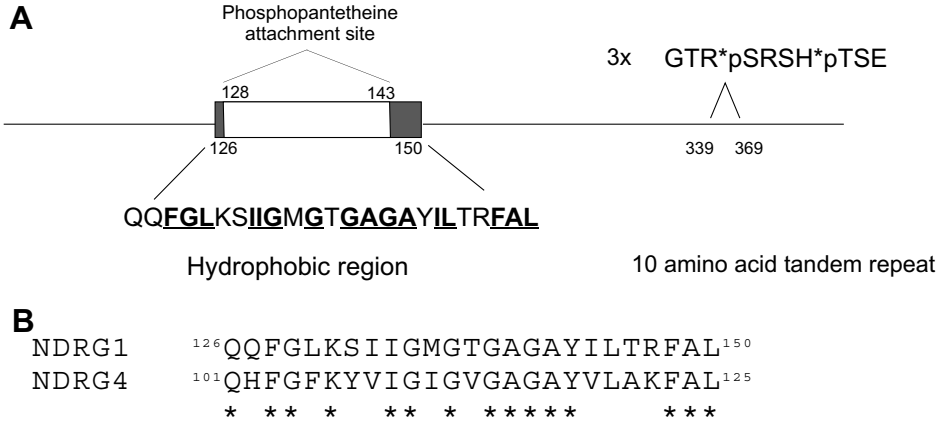

Fig. 3. Domain composition of NDRG1 gene. (A) Schematic representation of the putative domain composition of NDRG1. Hydrophobic amino acids are indicated in bold and underlined. F, G, L, I and A represent phenylalanine, glycine, leucine, isoleucine and alanine, respectively. The phosphorylated 10-amino acid tandem repeats located near the C-terminus are depicted. (B) Sequence homology of NDRG1 and NDRG4 hydrophobic regions.

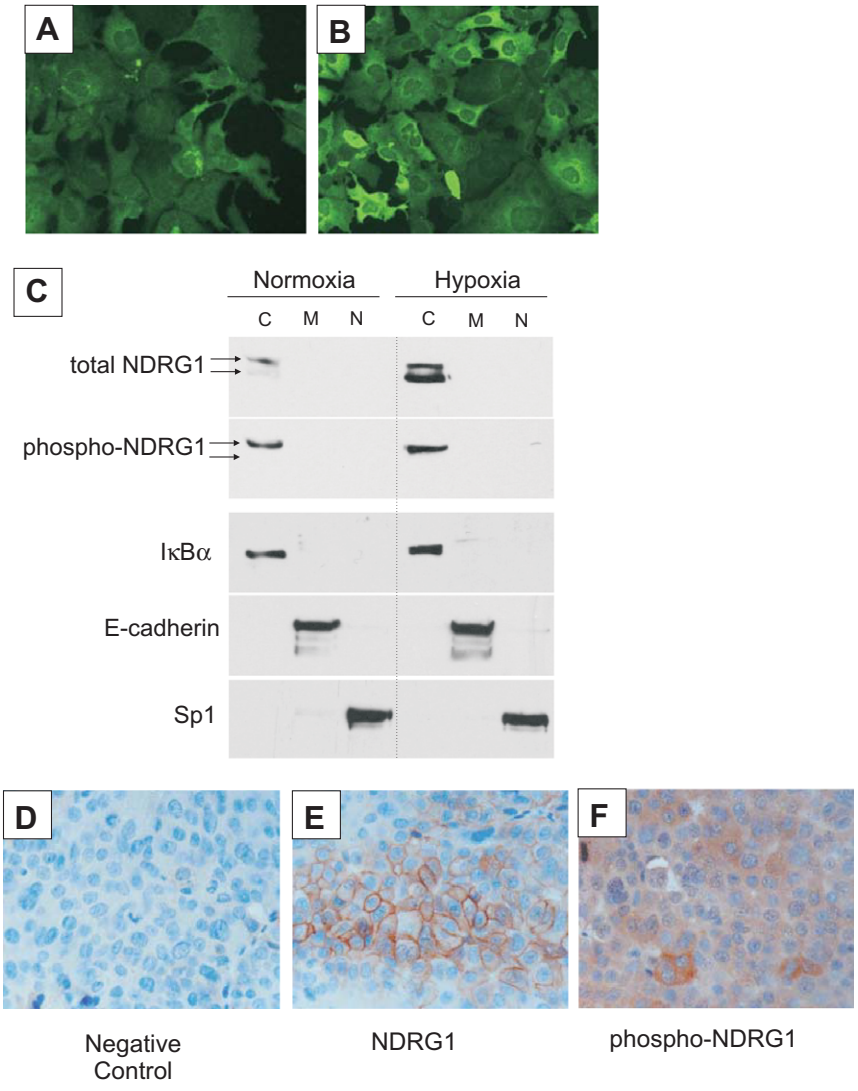

Fig. 4. Localization of NDRG1 in Hep3B cells in vitro and in vivo. Immunofluorescence NDRG1 expression in Hep3B cells grown in vitro under (A) normoxia, 21% O_2 or (B) hypoxia, 1.5% O_2 for 24 h. (C) Proteins were isolated by cell fractionation from Hep3B cells cultured in vitro. Antibodies against IκBα (40 kDa), E-cadherin (120 kDa) and Sp-1 (97 kDa) were used as markers for the cytoplasmic (C), the membrane (M) and the nuclear (N) fractions, respectively. (D-F) Immunohistochemistry detection of NDRG1 (E) and phosphorylated NDRG1 (F) in Hep3B cells grown in a mouse xenograft tumour in vivo. A negative control (D) illustrates the specificity of the reaction (200× magnification).

HCC-xenograft model in nude mice. Hep3B cells grown within an HCC-tumour environment showed increases in both the cytoplasmic and the membrane levels of NDRG1 (Fig. 4E). The pattern of expression in the murine xenografts was similar to that in human HCCs and the surrounding murine hepatic tissue displayed no immunoreactivity (data not shown). Furthermore using a phospho-specific antibody, we observed staining of phosphorylated NDRG1 predominately in the cytoplasm of the tumour cells in vivo (Fig. 4F). These findings demonstrate that NDRG1 can be translocated to the plasma membrane of Hep3B cells in vivo, but not under the tested signalling conditions in vitro. Furthermore, hypoxia-induced phosphorylation does not result in a membrane-associated phosphorylated protein in vivo or in vitro, thereby suggesting that tumour microenvironment harbours unidentified signals that are required for translocation of NDRG1 to the plasma membrane.

4. Discussion

Either transiently or chronically, hypoxic stress is a common characteristic of HCC tumours [22,23], therefore, we speculated that the upregulation of NDRG1 in HCC could be mediated via the hypoxic activation of the HIF signalling pathway. Here, we show in human HCC cells HIF-1α to be co-expressed with NDRG1 in vivo and under conditions that are known to activate HIF-1 in vitro (hypoxia or the presence of the prolyl hydroxylase inhibitor (DMOG)). Taken together, this suggests that one mechanism by which NDRG1 is overexpressed in HCC is the activation of the HIF-1 signalling pathway. This is in agreement with previous work, which proposed HIF-1 as a regulator of NDRG1 in human cancers [24].

In samples of human HCC which were analyzed by immunohistochemistry, NDRG1 expression was found in various subcellular compartments. Its expression was located both in the

cytoplasm and on the plasma membrane. A comparative sequence analysis yielded evidence for a putative membrane association of NDRG1 protein. The factors that contribute to the specificity of NDRG1 localization, and particularly to its membrane targeting, are currently unknown, thus leading us to examine the role of hypoxic stress. Our in vitro data confirms that hypoxia are a strong transcriptional upregulator of NDRG1 expression. The hypoxia-induced expression of total NDRG1 protein is followed by a subsequent increase of phorphorylated-NDRG1 on residues targeted by SGK1 [20]. Furthermore, after prolonged hypoxia (~12 h) there is also an increase in GSK activity, evident by the increased expression of the slower migrating phosphorylated band (Fig. 2B). This is consistent with the work of Mottet et al., who reported that prolonged hypoxia results in activation of GSK3 [25]. However, our data suggests that the hypoxia-induced increase of NDRG1 and phosphorylated NDRG1 is not sufficient for its membrane localization (Fig. 4C).

On the other hand, when cells were grown within an HCC-tumour environment in vivo, a strong membrane localization of total NDRG1 protein was observed. The membrane localization of total NDRG1 was not concomitant with its phosphorylated form. Phosphorylated NDRG1 was detected in the cytoplasm of Hep3B cells grown in vivo as well as in the cytoplasmic fraction of Hep3B cells cultured under hypoxic stress in vitro (Fig. 4C). A similar expression pattern of phosphorylated NDRG1 was observed from patient samples of HCC (data not shown). Although hypoxia is a key component of the tumour microenvironment and can lead to the increase of phosphorylated NDRG1, factors other than hypoxia are critically involved in the re-distribution of NDRG1. Its expression on the plasma membrane within a neoplastic milieu implies that NDRG1 may be involved in cell adhesion or cell mobility or it may have a role in signal transduction, acting as a signalling molecule regulated by phosphorylation. Further insight into the relationship between NDRG1 and the tumour stroma should improve our understanding of this protein's role in tumourigenesis.

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