Abstract

The transcriptional inhibitor Snail is a critical regulator for epithelial–mesenchymal transition (EMT). Although low oxygen induces Snail transcription, thereby stimulating EMT, a direct role of hypoxia-inducible factor (HIF) in this process remains to be demonstrated. Here we show that hypoxia induces the expression of Snail via HIF. In silico analysis identified a potential hypoxia-response element (HRE) close to the minimal promoter of the human and mouse genome of the snail gene. Gel shift assays demonstrated that a specific hypoxia-inducible complex is formed with the putative HRE and that the complex contains HIF proteins. ChIP assays confirmed the interaction of HIF proteins with the putative HRE in vivo. Reporter gene analyses showed that the putative HRE responds to hypoxia in its natural position as well as in front of a heterologous promoter and that the HRE is directly activated by HIF-1α or HIF-2α. HIF knockdown with siRNA at 2% oxygen and overexpression of an oxygen-insensitive HIF (HIF-ΔOEDIT) mutant at 21% oxygen showed that HIF regulates Snail activation.

Introduction

Hypoxia (low oxygen) is an important physiologic inducer of tumor metastasis. Epithelial–mesenchymal transition (EMT) is thought to be one of the initial steps in metastasis (1, 2). EMT converts cells from an epithelial, nonmotile morphology to become migratory and invasive (1, 2). Although many factors contribute to EMT, the transcriptional inhibitor Snail is considered to be a key EMT regulator (1, 2). Members of the Snail family are zinc-finger transcription factors that function as repressors of gene expression (3). Snail knockout mice die during gastrulation and show defects in EMT (4). In addition to their role in EMT, Snail-related proteins also participate in cell migration, left-right asymmetry (5), cell proliferation (6), and apoptosis (6, 7). Direct targets for Snail repression include E-cadherin, cytokeratin 18, and components of tight junctions such as claudins and occludin (3). Snail downregulates expression of E-cadherin, which plays a central part in the process of epithelial morphogenesis, and its loss stimulates cell migration and invasion. In contrast, Snail upregulates mesenchymal-produced fibronectin and vimentin (3) and molecules involved in the degradation of the basement membrane and extracellular matrix, such as metalloproteinas (8). Several signaling pathways regulating Snail transcription have been identified (3). Both mitogen-activated protein kinase (MAPK) and NFκB stimulate transcription through regions in the minimal promoter of Snail (9, 10) and many signaling molecules implicated in EMT progression use both signaling pathways to mediate their response (10–14). Other signaling pathways implicated in EMT, including Wnt and phosphoinositide 3-kinase (15, 16), use GSK-3 to mediate their responses. GSK-3 is an endogenous inhibitor of Snail transcription (17) and GSK-3β-mediated phosphorylation of Snail has been shown to facilitate its proteasomal degradation (18). Low oxygen induces Snail transcription (19–22) and both the canonical HIF (22, 23) and Notch (24, 25) pathways have been implicated in hypoxia-induced EMT. However, Snail is yet to be identified as a direct HIF target gene. HIF consists of heterodimers comprised of an alpha and beta subunit (26, 27). The regulation of HIF by oxygen occurs through modifications of the α-subunit, whereas the β-subunit is constitutively expressed and not affected by hypoxia (28). Hypoxic conditions allow the α-subunit to accumulate and translocate to the nucleus, where, upon binding to the β-subunit, it recognizes HIF-response elements containing the consensus sequence 5’-RCGTG-3’ within the promoter regions of hypoxia-responsive target genes. During normoxia, the α-subunit is rapidly hydroxylated by prolyl hydroxylase domain enzymes (29). The hydroxylated α-subunit is recognized by the von Hippel-Lindau E3 ubiquitin ligase complex and targeted for proteasomal degradation (29, 30).

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mechanistic insights into Snail regulation and hypoxia-induced EMT.

**Materials and Methods**

**Cell cultures**

The yolk sac endothelial C166 cells, human embryonic kidney 293T cells, human lung adenoma epithelial A549 cells and human choriocarcinoma epithelial JEG-3 cells were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% Fetal Bovine Serum. To mimic hypoxia, in some experiments, cells were exposed to 50 to 400 μM of CoCl2, concentrations that have previously been shown to efficiently induce HIF expression (31).

**Real-time PCR**

Cell cultures were washed with phosphate-buffered saline (PBS), RNA extracted with Trizol and reverse transcribed using random hexamers (Invitrogen). Real-time PCR was carried out as described previously (32) using primers for human **SNAIL**: 5’- primer GCTCGAAAGGCCTCAACTGCAAATACTGCAAC; 3’- primer GGGAGCAGGAGAAGGGCTTCTCGCCAGTGTG; or mouse **snail**: 5’- primer GCGGAAGATCTTCAACTGCAATTATTGTAAC; 3’- primer GCAGTGGGAGCAGGAAATGGCTTCAC.

**Nuclear protein extracts**

Nuclear extracts were prepared essentially as described previously (33). Cells (1 × 10^7) exposed to either 21% or 2% oxygen (or 200 μM CoCl2) for 6 hours were collected, washed in PBS and resuspended in 0.8 mL of ice-cold low salt buffer A [10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor mixture (Roche)]. After 10 minutes on ice, the nuclei were collected by centrifugation (10,000 × g for 2 minutes) and the pellet was resuspended in high salt buffer B [20 mM HEPES-KOH, pH 7.9, 20% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF and protease inhibitors]. Nuclei were incubated on ice for 30 minutes and centrifuged at 10,000 × g for 5 minutes to remove cell debris. The protein concentration of the nuclear extract was determined and small aliquots were frozen at –80°C until use.

**Western blot analysis**

Whole cell extracts were prepared by harvesting cells in radioimmunoprecipitation assay buffer followed by sonication. Equivalent amounts of nuclear and/or whole cell proteins were separated by 8% SDS-polyacrylamide gel electrophoresis under reducing conditions. After transfer onto polyvinylidene difluoride membrane, proteins were revealed with antibody of interest using enhanced chemiluminescence (Amersham). Primary antibodies were rabbit polyclonal anti-Snail (dilution of 1:800, Abcam Inc.), anti-vimentin (dilution of 1:500, Abcam Inc.), and anti-S100A4 (dilution of 1:1,000, Abcam Inc.) or mouse monoclonal anti-VE-Cadherin (dilution of 1:400, Santa Cruz Biotechnology), anti-E-cadherin (dilution of 1:500, Santa Cruz Biotechnology).

**Figure 1.** Hypoxia induces Snail mRNA expression in endothelial C166 cells. A cells were incubated under 2% O2 for indicated period of time (hours). Snail mRNA levels were determined by real-time RT-PCR. Fold increases were calculated as the ratio of Snail expression in 2% vs. 21% O2 exposed cells, both normalized to 18S RNA. B cells were treated with various concentrations of CoCl2 for 24 hours and Snail mRNA levels were determined. Fold increases were calculated as the ratio of Snail expression in CoCl2-treated cells vs. nontreated cells, both normalized to 18S RNA. Cells were treated with 200 μM CoCl2 for indicated period of time (days) and Snail mRNA levels were determined. Fold increases were calculated as the ratio of Snail expression in CoCl2-treated cells vs. nontreated cells, both normalized to 18S RNA. Data are mean ± S.E.M. from 3 independent experiments carried out in triplicate. *P < 0.05 vs. normoxia nontreated controls.
Biotechnology), and anti-HIF-1α (dilution of 1:1,000, mix of clones 7E3 and 7D6; ref. 34). Goat anti-rabbit or goat anti-mouse horseradish peroxidase—conjugated IgG were used as secondary antibodies (dilution of 1:10,000; Jackson ImmunoResearch, Cedarlane Laboratories Limited).

**Electrophoretic mobility shift assay**

Double stranded oligonucleotides with 5′-overhangs were labeled by incorporating [α-32P]dCTP with Klenow enzyme. Labeled probes (20,000 cpmp) were added to 20 μL binding reaction mixture (10 μL 2× binding buffer (20 mM HEPES-KOH, pH 7.9, 20% glycerol, 1 mM CaCl2, 10 mM MgCl2, 0.2 mM EDTA, 1 mM DTT), 0.8 μg poly d(IC) and 60 mM KCl) and then incubated with nuclear extracts (8 μg) for 15 minutes at room temperature. Competition assays were performed by adding a 10, 50, or 100 molar excess of unlabeled oligonucleotides to the binding reaction. In supershift experiments, HIF antibodies (Santa Cruz Biotechnology) were added to the binding reaction for an additional incubation of 30 minutes. The protein–DNA complexes were separated from the free probes on a 6% polyacrylamide gel of a low ionic strength [0.5× TBE (50 mM Tris–borate, 50 mM boric acid, 1 mM EDTA)] and dried gels were exposed for autoradiography. The following oligonucleotides were used (HRE core binding site bolded and underlined): (1) mouse snail-91: AGCCTTGACAAAGGGGCGTGACCAACAGTACGGTACGCCCCC, (2) mutated mouse snail-91: AGCCTTGACAAAGGGGAAAGACCAACAGTACGGTCACGCCCCC, and (3) EPO-HRE: GCCTACGTGCTGTCTAGACAGCCCTGCTGAC.

**Chromatin immunoprecipitation assay**

The chromatin immunoprecipitation (ChIP) assay was performed as described by Weinmann and Farnham (35), with minor modifications. C166 cells were fixed at 37°C for

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**Figure 2.** Hypoxia triggers epithelial/endothelial mesenchymal transition. Cells (A: C166 cells, B: A549 cells, and C: JEG-3 cells) exposed for 5 days to either 2 or 21% O2 were analyzed by either Western blotting (A: Snail, VE-cadherin, S100A4 and B: E-cadherin, vimentin) or immunofluorescence (C: E-cadherin). Data shown are a representative of at least 3 experiments.

**Figure 3.** Analysis of the human and mouse Snail genomic structure. A schematic illustration of human and mouse Snail promoter. B two conserved putative response sequences close to the start site of transcription (numbering relative to transcription start site (labeled +1) of Snail) have inverted repeats and perfect homology to the consensus HRE sequence (−A/GCGTG−).
10 minutes in 1% formaldehyde. Cells were then collected, nuclei were isolated by incubating the cells for 10 minutes on ice in cell lysis buffer (10 mM Tris, 10 mM NaCl, 0.2% Nonidet P-40, pH 8.0) containing protease inhibitors and PMSF. Nuclei were collected by centrifugation and then lysed in ice-cold nuclei lysis buffer (50 mM Tris, 10 mM EDTA, 1% sodium dodecyl sulfate (SDS), 10 mM sodium butyrate, protease inhibitors, 50 μg/mL PMSF, pH 8.1). Chromatin was broken into relatively small fragments by sonicating the nuclear lysate on ice in fragments of an average size of 500 to 1,000 bp (6 to 8 pulses of 15 seconds at 80% of maximum power using a Misonix Sonicator 3000 equipped with a microtip). Samples were precleared with protein G-coated agarose beads (Santa Cruz Biotechnology) in IP wash buffer I (20 mM Tris, 50 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, pH 8.1). The precleared supernatants were aliquoted for subsequent immunoprecipitation. Equal aliquots of precleared chromatin samples were incubated overnight with either specific rabbit anti-HIF-1α (ABR Affinity BioReagents or Santa Cruz Biotechnology) or anti-HIF-2α (Santa Cruz Biotechnology) antibodies or nonspecific rabbit antiserum at 4°C. An additional aliquot of chromatin sample, 20% of the volume of other aliquots (input), was saved for the subsequent PCR analysis. Immune complexes were collected by incubation with 50 μL protein G coated agarose beads for 2 hours at 4°C. The pellets were washed 3 times with 500 μL of IP wash buffer I and twice with IP wash buffer II (10 mM Tris, 0.25 M LiCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, pH 8.1). Immune complexes were eluted from the beads by incubation twice under agitation at 37°C with 150 μL of IP elution buffer (0.1 M NaHCO3, 1% SDS). The eluted material was washed 3 times with 500 μL of IP wash buffer I and twice with IP wash buffer II (10 mM Tris, 0.25 M LiCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, pH 8.1). Immune complexes were collected by incubation twice under agitation at 37°C with 150 μL of IP elution buffer (0.1 M NaHCO3, 1% SDS). The eluted material was pooled, RNase A (3 μg/mL) and NaCl (0.3 M) were added, and cross-linking was reversed by incubation for 4 to 5 hours at 65°C. Samples were digested with proteinase K (0.2 mg/mL) for 2 hours at 45°C. Immunoprecipitated DNA was purified by extraction with phenol/chloroform followed by ethanol precipitation. Purified immunoprecipitated DNA

Figure 4. Electrophoretic gel shift analysis. A binding of hypoxia-inducible transcription factors to putative ms-91-HRE. Lane 1: Free 32P-labeled ms-91-HRE oligonucleotide probe; lane 2: 32P-labeled ms-91-HRE oligo incubated with nuclear extract from 21% O2 treated C166 cells; lane 3: 32P-labeled ms-91-HRE oligo plus nuclear extract from 2% O2 treated C166 cells; lanes 4–6: 32P-labeled ms-91-HRE oligo incubated with nuclear extract from 2% O2 in the presence of 10-, 50-, and 100-fold molar excess of cold ms-91-HRE probe (lanes 4–6) or 100-fold molar excess of either cold ms-91-HRE with mutation in both of the HRE core sequences (lane 7) or a cold probe with the EPO-HRE consensus sequence (lane 8). B presence of HIF-1α in hypoxia-inducible complex. Lane 1: 32P-labeled ms-91-HRE oligo plus nuclear extract from 2% O2 treated C166 cells; lanes 2 and 3: 32P-labeled ms-91-HRE oligo incubated with nuclear extract from 2% O2 treated C166 cells in the presence of either 100-fold molar excess of cold ms-91-HRE probe (lane 2) or HIF-1α specific antibody (lane 3); lanes 4 and 5: 32P-labeled mutated ms-91-HRE incubated with nuclear extract from 2% O2 treated C166 cells in absence (lane 4) or presence of 100-fold molar excess of ms-91-HRE (lane 5); lanes 6 and 7: 32P-labeled EPO-HRE incubated with nuclear extract from 2% O2 treated C166 cells in absence (lane 6) or presence of HIF-1α specific antibody (lane 7). Data are representative of 3 separate experiments.
and input DNA were resuspended in 50 and 100 μL of water, respectively. Three microliters of DNA was amplified by PCR with a temperature cycle of 30 seconds at 94°C, 30 seconds at 65°C, and 25 seconds at 72°C for 32 cycles. Primers used for PCR correspond to the mouse snail promoter region (−120/+150 relative to transcription start site), primers: 5′-GCGGTACCTTGACAAAGGGGCGTGACCAACAGTACGGTTAC-3′; 3′ primer: 5′-GGCGAAGCCTTGACCCACCAC-3′. As a positive control the mouse VEGF-HRE region was amplified using the following primers: 5′-GCGGTACCTTGACAAAGGGGCGTGACCAACAGTACGGTTAC-3′; 3′ primer: 5′-AGCGCCCTTGTCCGACCTGC-3′. A region of mouse snail gene downstream of the HRE sequence (−150 to −120) of Snail promoter (upper panel), a 380 bp fragment encompassing mouse VEGF-HRE region (middle panel), or a sequence downstream of the ms-91-HRE (−350 to −300) with a size of 260 bp (lower panel). Data are representative of 2 separate experiments.

Expression vector

Human HIF-1α and HIF-2α cDNAs were reverse transcribed from total RNA isolated from JEG-3 cells treated with hypoxia. The cDNAs were then PCR-amplified to generate only the coding regions, with primers containing Kpn I and Not I restriction sites at 5′ and 3′ ends. The purified PCR products were digested with Kpn I and Not I and ligated into pcDNA3.1 plasmid digested with the same restriction enzymes. In addition, isolated mouse Snail-HRE [restriction sites underlined; HRE core binding site bolded and underlined] GCGGTACCTTGACAAAGGGGCGTGACCAACAGTACGGTTAC-3′; 3′ primer: 5′-GGCGAAGCCTTGACCCACCAC-3′. As a positive control the mouse VEGF-HRE region was amplified using the following primers: 5′-GCGGTACCTTGACAAAGGGGCGTGACCAACAGTACGGTTAC-3′; 3′ primer: 5′-AGCGCCCTTGTCCGACCTGC-3′. A region of mouse snail gene downstream of the HRE sequence (−150 to −120) of Snail promoter (upper panel), a 380 bp fragment encompassing mouse VEGF-HRE region (middle panel), or a sequence downstream of the ms-91-HRE (−350 to −300) with a size of 260 bp (lower panel). Data are representative of 2 separate experiments.

Figure 5. Chromatin immunoprecipitation assays. C166 cells were exposed for 6 hours to 21% O2 [lanes 1–10] and 2% O2 (lanes 5–10) oxygen. Rabbit anti-HIF-1α (lanes 3, 8 (ABR), and 4, 9 (Santa Cruz)), anti-HIF-2α (lanes 5 and 10) and nonimmune (lanes 2 and 7) antisera were used for immunoprecipitation. Immunoprecipitated and purified DNA together with 1% of input DNA (lane 1 for normoxia, lane 6 for hypoxia) were used for PCR amplification of either a 270 bp product encompassing ms-91-HRE region (−120 to +150) of Snail promoter (upper panel), a 380 bp fragment encompassing mouse VEGF-HRE region (middle panel), or a sequence downstream of the ms-91-HRE (−350 to −300) with a size of 260 bp (lower panel). Data are representative of 2 separate experiments.

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Reporter constructs

SEAP2 constructs containing various lengths of mouse Snail promoter were generated by PCR amplification from a 1.8 kb Kpn I/Hind III genomic template and insertion into a SEAP2 reporter plasmid (pSEAP2-Basic, Clontech Laboratories, Inc.). Sequences of the 5′-primers were (restriction sites underlined): pB1.2kb: 5′-GCGGTACCATCGATGCCCTAGAGACCTGAATGCTAGGGTGGTTA-3′; pB0.2kb: 5′-GCGGTACCATCGATGCCCTAGAGACCTGAATGCTAGGGTGGTTA-3′. A region of mouse snail gene downstream of the HRE sequence (−150 to −120) of Snail promoter (upper panel), a 380 bp fragment encompassing mouse VEGF-HRE region (middle panel), or a sequence downstream of the ms-91-HRE (−350 to −300) with a size of 260 bp (lower panel). Data are representative of 2 separate experiments.
between promoter (SEAP) activities in cells cultured at 2 vs. 21% O2, both (SEAP) activities were measured. Fold induction was measured as the ratio cells were incubated for 24 hours in 2% or 21% oxygen and promoter transiently transfected into C166 (B) or 293T (C) cells. After transfection, www.aacrjournals.org Mol Cancer Res; 9(2) February 2011

mutated pSnail-mtHRE-SV40-SEAP2, p(Snail-HRE)2-SV40-SEAP2, and constructs: control vector pSV40-SEAP2, pSnail-HRE-SV40-SEAP2, heterologous promoter. A schematic illustration of Snail HRE reporter Figure 6.

positive control p(EPO-HRE)2-SV40-SEAP2. B, C reporter constructs were 5 experiments in triplicate. * normalized to pSV40-SEAP2 control vector. Data are mean

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ACACAGCCTGTCTGAGCCTACGTGCTGCTCAGACAGCCTGTCTCGAGCAGGCCG (in tandem), were cloned into the Kpn I/Xho I restriction site of the pSEAP2-Promoter (Clontech Laboratories, Inc.) containing the SV40 promoter.

Transient transfection and SEAP2 assay

293T cells in 24-well culture plates were transiently transfected using lipofectamine 2000 (Invitrogen Canada Inc.) with 0.5 μg of pSEAP2 reporter plasmids and 0.02 μg of β-galactosidase plasmid as an internal control for transfection efficiency. In cotransfection experiments, cells were cotransfected with pcDNA3.1 empty vector, pcDNA-HIF-1α or pcDNA-HIF-2α expression plasmids. The total amount of DNA transfected in each culture well was made up to 0.5 μg with pcDNA3.1 plasmid DNA. AMAXA Nucleofector electroporation (Lonza-Cedarlane Laboratories) was used for introduction of plasmid DNA into C166 cells. The cell-type-specific Nucleofector Solution used was type V and the electrical parameter of the Nucleofector used for C166 cells was T-30. A total of 3 μg plasmid was used to transfect 1.8 million cells. After electroporation, cells were cultured in 10% FBS-DMEM either in 6-well plates for isolation of proteins for Western blotting analyses or in 24-well culture plates for SEAP2 assay and continue the following steps. The medium was removed after 6 to 12 hours and fresh culture medium was added. Cells were incubated for another 24 hours before 50 μL of culture supernatant was collected. Cell debris was removed by centrifugation and SEAP2 activities were measured using the Phospho-Light TM reagent as outlined by the manufacturer (Tropix Applied Biosystems). Light intensity was measured in an EG&G Berthold luminometer. All transfections were performed in triplicates and repeated at least 4 times.

Cell migration assay

Transwell polycarbonate membrane inserts (6.5 mm) with a 8.0 μm pore size (Corning Inc. Life Sciences) and precoated with 2.5 μg/cm² fibronectin were used in the migration assay. Expression plasmids (pcDNA3.1 empty vector and pcDNA-HIF-1α-ODD), or Dharmacon ON-TARGETplus SMARTpool siRNAs (targeting mouse HIF-1α, HIF-2α or a scrambled sequence negative control) were introduced into C166 cells by Nucleofector electroporation as described above. Transfected and nontransfected C166 cells were incubated for 48 hours in 37°C at 5% CO2 under 2% or 21% O2 with serum-free DMEM. Cells were then trypsinized, counted and 2 × 10⁵ cells in 0.2 mL serum-free DMEM were loaded onto Transwell inserts in the upper chamber of 24-well Transwell plate with the bottom chamber filled with 680 μL of DMEM supplemented with 5% FBS. Cells were left to migrate for 16 hours at 37°C under 2% or 21% O2. Cells were then fixed in 4% paraformaldehyde for 15 minutes, stained with hematoxylin for 1 minute and nonmigrated (upper chamber side) cells were removed by scraping with a wet cotton swab. The number of cells that migrated across the filter was counted
in 4 high-power fields per insert and average values were calculated. For each migration condition, 3 identical replicates were performed.

Data analysis
Data are presented as mean ± S.E.M. Statistical significance was determined by one-way analysis of variance followed by assessment of differences using Student–Newman–Keuls test. Statistical analysis was performed with the JMP statistical software and significance was accepted at \( P < 0.05 \).

Results

Hypoxia increases Snail expression and triggers EMT
Endothelial C166 cells were exposed to 2% \( O_2 \) or treated with \( \text{CoCl}_2 \) to mimic hypoxia (31). Hypoxic conditions significantly increased Snail mRNA expression when compared to normoxic controls (Fig. 1A–C). Dose–response experiments showed that induction of Snail transcription was optimal with 0.2 to 0.3 mM of \( \text{CoCl}_2 \) (Fig. 1B). Time-course experiments revealed that hypoxic induction of Snail transcription peaked around 4 to 24 hours and declined thereafter (Fig. 1A and C). Similar hypoxia-induced increases in Snail expression were observed in A549 and JEG-3 cells (not shown). Since Snail is implicated in epithelial (endothelial) mesenchymal transition [E(En)MT] we investigated whether hypoxia would trigger E(En)MT in C166, A549, and JEG-3 cells. Western blot analysis of C166 cells showed that hypoxic exposure of 5 days increased Snail protein levels, confirming the real-time PCR results. This increase in Snail expression was accompanied by a decrease in endothelial VE-cadherin and an increase in mesenchymal S100A4 expression (Fig. 2A). Hypoxia reduced E-cadherin levels of epithelial A549 (Fig. 2B) and JEG-3 (Fig. 2C) cells, while expression of vimentin, a mesenchymal marker, increased (Fig. 2B). These alterations in expression patterns are typical of E(En)MT.

Hypoxia-inducible proteins bind to a putative HRE within the minimal Snail promoter
In silico analyses of human and mouse Snail gene identified multiple potential hypoxia-response elements (Fig. 3). Special attention was given to 2 conserved putative response sequences close to the start site of transcription which were 100% homologous to the consensus HRE sequence (-A/GCGTG-). Since our preliminary experiments showed that the downstream HRE consensus sequence was not a functional HRE (data not shown), we focused our efforts on the upstream HRE element located -91bp of mouse snail gene. Gel shift assays were carried out with a double-stranded \(^{32}\text{P}\)-radiolabelled oligodeoxyribonucleotide probe containing the putative hypoxia-responsive element using nuclear extracts from either control or hypoxia exposed C166 cells (Fig. 4A and B). No specific complex was detected using nuclear extracts from cells cultured at 21% \( O_2 \) (Fig. 4A, lane 2). However, a strong binding activity is shown in lane 3, 6 and 7, which correspond to co-transfection with pcDNA3-HIF-1\( \alpha \) or pcDNA3-HIF-2\( \alpha \), respectively. A specific complex is also detected in lane 5 which corresponds to transient transfection with pcDNA3-HIF-1\( \alpha \) and HRE element located -91bp of mouse snail gene. Gel shift assays were carried out with a double-stranded \(^{32}\text{P}\)-radiolabelled oligodeoxyribonucleotide probe containing the putative hypoxia-responsive element using nuclear extracts from either control or hypoxia exposed C166 cells (Fig. 4A and B). No specific complex was detected using nuclear extracts from cells cultured at 21% \( O_2 \) (Fig. 4A, lane 2). However, a strong binding activity is shown in lane 3, 6 and 7, which correspond to co-transfection with pcDNA3-HIF-1\( \alpha \) or pcDNA3-HIF-2\( \alpha \), respectively. A specific complex is also detected in lane 5 which corresponds to transient transfection with pcDNA3-HIF-1\( \alpha \) and HRE element located -91bp of mouse snail gene.

Figure 7. Effect of HIF proteins on ms-91-HRE in the context of a heterologous promoter. Reporter constructs (see Fig. 6A) together with either pcDNA3-HIF-1\( \alpha \), pcDNA3-HIF-2\( \alpha \), or empty pcDNA3.1 vector were transiently transfected into 293T or C166 cells, incubated for 24 hours in 2% oxygen and promoter (SEAP) activities were measured. A HIF reporter plasmid contains the mouse hypoxia-responsive element (HRE) -91bp of the mouse snail gene. Gel shift assays were carried out with a double-stranded \(^{32}\text{P}\)-radiolabelled oligodeoxyribonucleotide probe containing the putative hypoxia-responsive element using nuclear extracts from either control or hypoxia exposed C166 cells (Fig. 4A and B). No specific complex was detected using nuclear extracts from cells cultured at 21% \( O_2 \) (Fig. 4A, lane 2). However, a strong binding activity is shown in lane 3, 6 and 7, which correspond to co-transfection with pcDNA3-HIF-1\( \alpha \) or pcDNA3-HIF-2\( \alpha \), respectively. A specific complex is also detected in lane 5 which corresponds to transient transfection with pcDNA3-HIF-1\( \alpha \) and HRE element located -91bp of mouse snail gene. Gel shift assays were carried out with a double-stranded \(^{32}\text{P}\)-radiolabelled oligodeoxyribonucleotide probe containing the putative hypoxia-responsive element using nuclear extracts from either control or hypoxia exposed C166 cells (Fig. 4A and B). No specific complex was detected using nuclear extracts from cells cultured at 21% \( O_2 \) (Fig. 4A, lane 2). However, a strong binding activity is shown in lane 3, 6 and 7, which correspond to co-transfection with pcDNA3-HIF-1\( \alpha \) or pcDNA3-HIF-2\( \alpha \), respectively. A specific complex is also detected in lane 5 which corresponds to transient transfection with pcDNA3-HIF-1\( \alpha \) and HRE element located -91bp of mouse snail gene.
was observed with nuclear extracts from C166 cells exposed to 2% O₂ for 6 hours (Fig. 4A, lane 3; Fig. 4B, lane 1). The specificity of this protein-oligonucleotide interaction was demonstrated in experiments on which increasing amounts of cold ms-91-HRE (Fig. 4A, lanes 4–6; Fig. 4B, lane 2) or cold EPO-HRE oligos (Fig. 4A, lane 8) were added to the binding reaction. Excess of cold oligos efficiently outcompeted the hypoxia-induced 32P-radiolabelled complex formation. In contrast, no competition was observed with 100-fold excess of cold ms-91-mtHRE, an oligonucleotide that had been mutated by converting 3 nucleotides of both core HRE sequences (Fig. 4A, lane 7). Consistent with the latter observation, no complex was observed using ms-91-mtHRE oligonucleotide as a probe (Fig. 4B, lanes 4, 5). When a specific HIF-1α antibody was added to the binding reaction, the hypoxia-inducible complex was partially eliminated and supershifted (Fig. 4B, lane 3), indicating that HIF-1α is a component of the complex. Similar observations were made with a 32P-radiolabelled EPO-HRE probe (Fig. 4B, lanes 6, 7).

**HIF proteins bind to the mouse Snail promoter**

To demonstrate that HIFs can bind to the mouse Snail promoter in vivo, ChIP assays were performed. Endothelial C166 cells cultured for 6 hours at either 2% or 21% O₂ were fixed with formaldehyde and cell nuclei were isolated and sonicated. The sonicated chromatin lysates were immunoprecipitated with either HIF-1α or anti-HIF-2α antibodies. Extracted DNA was used to amplify a 270 bp fragment of mouse Snail promoter (~120 to ~150). The amplified 270 bp product was only observed in DNA extracted from hypoxia-exposed samples immunoprecipitated with either HIF antibody (Fig. 5, lanes 8 to 10). No product was amplified when nonspecific rabbit antiserum was used (antibody control, Fig. 5, lanes 7). Primers of the mouse VEGF-HRE region amplified a 380 bp fragment (positive control) in a similar pattern as seen for the ms-91-HRE target gene while a sequence downstream of the ms-91-HRE with a size of 260 bp showed no specific amplification under hypoxic condition. These results corroborate our gel shift assay findings and show that HIFs bind to the Snail promoter sequence encompassing the ms-91-HRE under hypoxic conditions.

**Mouse Snail HRE responds to hypoxia via HIF**

A single copy of the 35-bp ms-91-HRE and its mutated counterpart were cloned upstream the SV40 promoter driving the SEAP2 reporter gene. In addition, 2 ms-91-HREs linked in tandem and 2 EPO-HREs in tandem were cloned upstream into the SV40-SEAP2 expression vector (Fig. 6A). Both 293T and C166 cells were transfected with the SEAP2 reporter constructs together with a β-galactosidase expression vector to normalize for transfection efficiencies. Fold increase in SEAP2 activity elicited by 2% O₂ exposure was determined for each construct relative to normoxic (control) SEAP2 activity. After 24 hours of hypoxia treatment of both cell lines SEAP activity increased 3- to 6-fold for the single ms-91-HRE containing construct
and 4- to 8-fold for the 2 copy version compared to normoxic controls (Fig. 6B and C). The SV40-SEAP2 construct with the mutated ms-91-HRE was not responsive to hypoxia. In contrast, hypoxia increased SEAP activity of the control vector containing 2 HREs of the erythropoietin gene by 5- to 9-fold (Fig. 6B and C). These findings demonstrate that the ms-91-HRE is a functional HRE acting as a hypoxia-enhancer regardless of cell type. To test whether HIF overexpression would enhance SEAP activity as seen with 2% O₂, we performed co-transfection experiments in 293T and C166 cells. The HRE containing reporter constructs were cotransfected with expression vectors encoding HIF-1α, HIF-2α, or empty vector pcDNA3.1, respectively. Western blot analyses of nuclear extracts from the cotransfected cells demonstrated high levels of HIF protein expression in the transfected cells (Fig. 7A). Both HIF-1α and HIF-2α-induced transcriptional SEAP activation in cells transfected with ms-91-HRE or EPO-HRE containing constructs (Fig. 7B and C). Thus, in the context of a heterologous promoter, ms-91-HRE is activated by HIF.

Mouse Snail HRE functions in the native promoter

To test the hypoxia responsiveness of the −91 to −83 HRE region in its natural location, we cloned 0.2 and 1.2 kb fragments of the 5′-flanking region of the mouse snail gene, encompassing the HRE and native minimal promoter, into a promoterless SEAP2 reporter vector (Fig. 8A). The fragments were obtained by PCR amplification of a 1.8 kb mouse genomic construct containing the whole Snail promoter region. The 0.2 kb fragment, encompassing −91 to +150, relative to the transcription start site, was used to create a reporter construct containing 2 mutations in the 2 core sequences of the HRE. The reporter constructs were transfected into C166 or 293T cells. Six hours after transfection, cells were incubated for 24 hours in 2% O₂. Again, fold induction was measured as the ratio between promoter activities in cells cultured under hypoxic (2% O₂) and normoxic (20% O₂) conditions. Hypoxia treatment resulted in a 4- and 10-fold transcriptional activation in C166 and 293T cells, respectively, transfected with either pcDNA3-HIF-1α, pcDNA3-HIF-2α, or empty pcDNA3.1 vector were transiently transfected into C166 (A) or 293T cells (B), incubated for 24 hours in 2% oxygen and promoter (SEAP) activities were measured. The fold of induction was measured as the ratio between promoter activities in cells transfected with HIF or empty expression vector. Data are mean ± S.E.M., n = 4 experiments in triplicate. *P < 0.05 vs. control SV40-SEAP2 vector (cotransfected with pcDNA3-HIF-1α); #P < 0.05 vs. control SV40-SEAP2 vector (cotransfected with pcDNA3-HIF-2α).

As shown (Fig. 10A) overexpression of an oxygen-insensitive HIF-1α (HIF-1αΔODD) at 21% O₂ increased Snail levels. A downstream target of Snail, S100A4, increased parallel to Snail. Knock-down of endogenous HIF-1α by siRNA under hypoxic conditions attenuated the increase of Snail and S100A4 (Fig. 10B). Similar results were obtained with HIF-2α siRNA. To determine whether the hypoxia-induced changes in Snail expression caused phenotypic alterations in C166 cells, we performed cell migration assays. Low oxygen

Figure 9. Effect of HIF proteins on native Snail promoter activity. Reporter constructs (see Fig. 8A) together with either pcDNA3-HIF-1α, pcDNA3-HIF-2α, or empty pcDNA3.1 vector were transiently transfected into C166 (A) or 293T cells (B), incubated for 24 hours in 2% oxygen and promoter (SEAP) activities were measured. The fold of induction was measured as the ratio between promoter activities in cells transfected with HIF or empty expression vector. Data are mean ± S.E.M., n = 4 experiments in triplicate. *P < 0.05 vs. control SV40-SEAP2 vector (cotransfected with pcDNA3-HIF-1α); #P < 0.05 vs. control SV40-SEAP2 vector (cotransfected with pcDNA3-HIF-2α).

Effect of HIF proteins on native Snail promoter activity.

Induction of Snail by HIF promotes endothelial cell migration

To demonstrate that Snail is directly regulated by HIF, experiments were performed to show that (1) overexpression HIF-1α under normoxia upregulates Snail and (2) knockdown of HIF under hypoxia downregulates Snail expression.
increased migration of C166 cells which was halved by downregulation of either endogenous HIF-1α or HIF-2α with siRNA (Fig. 10C). Overexpression of HIF-1α-ODD under normoxic conditions stimulated migration of C166 cells (Fig. 10D). Together these data indicate that HIF directly induces Snail expression in mouse C166 cells and promotes cell migration.

Discussion

In this report, we demonstrate that hypoxia induces the expression of Snail via HIF. This conclusion is based on 4 observations: first, in silico analysis identified a potential HRE in the Snail promoter; second, gel shift, and ChIP assays demonstrated interaction of HIF proteins with the putative HRE; third, the HRE is a functional cis-acting element that responds to hypoxia and is activated by HIF proteins; and fourth, knockdown of HIF attenuates hypoxia-induced Snail activation and cell migration while overexpression of HIF-ΔODD at normoxia stimulates Snail expression and cell migration.

Numerous signaling pathways have been implicated in the regulation of Snail expression during development. Snail expression is activated via fibroblast growth factor receptor 1 signaling during mammalian gastrulation (10). Transforming growth factor-β increases Snail expression during hair bud morphogenesis (37), while Notch upregulates Snail during cardiac valve development (38). Studies with cultured cells have identified several intracellular signaling pathways capable of inducing Snail transcription, including the MAPK, phosphoinositide-3-kinase (PI3K), integrin-linked kinase (ILK), and nuclear factor-κB (NFκB) pathways (39–42). Several transcription factors have been linked to Snail induction, including Gli1 (43), early growth response 1 (EGR1; ref. 44), high mobility group AT-hook 2 (45), and NFκB (40, 41). Response elements to some of

Figure 10. Induction of Snail expression by HIF promotes C166 cell migration. A C166 cells were transiently transfected with pcDNA3-HIF-1α-ODD or empty pcDNA3.1 vector, incubated for indicated time periods in 21% O2 and protein extracts were analyzed by Western blotting. B C166 cells exposed to siRNAs targeting either mouse HIF-1α, HIF-2α, or a scrambled sequence (negative control) were incubated for 48 hours under 2% or 21% O2 and protein extracts were analyzed by Western blotting. C nontransfected or siRNA transfected C166 cells were incubated for 48 hours at 2% (open bar) or 21% O2 (closed bar) with serum-free DMEM prior to being loaded in the upper chamber of Tanswell inserts with the bottom chamber filled with 5% FBS. D nontransfected and pcDNA3-HIF-1α-ODD transfected cells were incubated for 48 hours at 21% O2 in serum-free DMEM prior to being loaded in the upper chamber of Tanswell inserts with the bottom chamber filled with 5% FBS. Cells (C, D) were left to migrate for 16 hours and 4 high-power fields per insert were counted. Data are mean ± S.E.M., n = 3 replicates. Data are representative of 2 separate experiments. (C) *P < 0.05 vs. 21% O2, #P < 0.05 vs. nontransfected control at 3% O2; (D) *P < 0.05 vs. nontransfected control.
the above pathways have been identified in the 5’ proximal promoter region of the snail gene. Recent data also suggests epigenetic regulation of Snail. Palmer and colleagues (46) identified an enhancer element inside a robust DNase hypersensitive site (HS) in A375 melanoma cells. The element is located in the 3’ region of the snail gene and is conserved throughout the mammalian lineage. Activity of this enhancer is associated with enrichment of H3 Lys (4) dimethylation and H3 acetylation of both the enhancer and promoter. Although hypoxia has been implicated in upregulation of Snail (19–21), no functional hypoxia-responsive element has been identified in the snail promoter.

Various reports have suggested a link via Snail between hypoxia and EMT. Imai and colleagues (19) reported that mRNA expression of Snail was increased under hypoxia in ovarian cancer cell lines. In addition, they demonstrated that hypoxia downregulated E-cadherin in these cells. Lester and colleagues (47) showed that breast cancer cells cultured in low O2 exhibited changes consistent with EMT such as loss of E-cadherin from plasma membranes. Conversely, Snail expression was increased in these breast cancer cells. Storci and colleagues (48) reported that expression of Slug, another member of the Snail gene family of zinc-finger transcription factors, in tumor tissues correlated with that of the hypoxia survival gene carbonic anhydrase IX. Furthermore, exposure of sarcoma MCF-7 cells to a hypoxic environment led to upregulation of the Slug gene (48). Others have found that stabilization of HIF due to loss of von Hippel-Lindau (VHL) protein leads to repression of E-cadherin expression. VHL-defective renal carcinoma RCC cells have elevated expression of well-known E-cadherin-specific repressors including Smad-interacting protein-1 (SIP1, also known as ZEB-2 and ZFHX1B) and Snail (49). Modest increases in TCF-3 and dEF1 (ZFHX1A) repressors were found (50). While these repressors act on the conserved E2 boxes located within the promoter region of E-cadherin, no reports have provided a mechanism for hypoxia-increased expression of Snail. In the present study, we demonstrate that HIF binds to a HRE cis-acting element in the promoter region of the snail gene that activates its transcription and subsequently suppresses the expression of E-cadherin and promotes migration.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Hypoxia Induces Snail Expression via HIF-1


Mouse Snail Is a Target Gene for HIF
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