Published in final edited form as:

Methods Enzymol. 2007 ; 435: 355–369. doi:10.1016/S0076-6879(07)35018-0.

Macrophage Migration Inhibitory Factor Manipulation and Evaluation in Tumoral Hypoxic Adaptation

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Abstract

Increasingly clear is an important regulatory role for hypoxia-inducible factor 1 α (HIF-1 α) in the expression of the cytokine/growth factor macrophage migration inhibitory factor (MIF). The functional significance of hypoxia-induced MIF expression is revealed by findings demonstrating that HIF-1 α -dependent MIF expression is necessary for hypoxia-induced evasion from cell senescence and that MIF is necessary for HIF-1 α stabilization induced by hypoxia and prolyl hydroxylase (PHD) inhibitors. Both of these activities attributed to MIF likely involve the modulation of protein degradatory pathways mediated by cullin-dependent E₃ ubiquitin ligase complexes and their regulation by the COP₉ signalosome (CSN). As the importance of MIF in hypoxic adaptation of human tumors is now becoming fully realized, we review protocols designed to evaluate MIF expression, activity, and functional consequences in hypoxic environments.

1. Introduction

Since the cloning of the factor responsible for a transcriptional activity associated with hypoxic adaptation (Wang *et al.*, 1995), the molecular events involved in the degradation of HIF- α have been extensively characterized. These studies led to the eventual identification of a family of PHDs that serve to act as dioxygen sensors (Kim and Kaelin, 2003). Under normoxic conditions, HIF-1 α undergoes trans-4-hydroxylation at Pro-564 (C_{ODD}, or C-terminal ODD) and Pro-402 (N_{ODD}, or N-terminal ODD), which form part of highly conserved LXXLAP motifs in oxygen-dependent degradation domains (ODDs) (Chan *et al.*, 2005; Kim and Kaelin, 2003). Hydroxylation allows recognition of HIF-1 α by the von Hippel-Lindau tumor suppressor protein (pVHL), which serves as the recognition component of the E3 ubiquitin ligase complex consisting of VHL/elongin C/elongin B (VCB), cullin 2, and the RING-H2 finger protein Rbx-1 (Hon *et al.*, 2002). Importantly, structural analysis of the HIF-C_{ODD} and pVHL reveals that all five pVHL residues lining the 4-hydroxyproline-binding pocket are affected by missense mutations in VHL disease (Kim and Kaelin, 2004). This characteristic suggests that failure to capture HIF-1 α and/or other hydroxylated targets is important to the tumor-promoting mechanism associated with VHL disease. Subsequent ubiquitylation of HIF- α by the Cdc34/Ubc5 E2 ubiquitin-conjugating complex targets HIF- α for transport to the proteasome and degradation.

COP₉ signalosome subunit 5 (CSN5) is an essential component of CSN, which is composed of eight subunits designated CSN1 to CSN8 (Wolf *et al.*, 2003). Until recently, the function of the CSN was obscure, though it appeared to control proteins that had high turnover rates. The conjugation of the small ubiquitin-like protein Nedd8 to cullins is thought to be required for E2 recruitment and targeted ubiquitylation. CSN5 contains a JAB-1/MPN domain metalloenzyme motif (JAMM), which forms the catalytic region of the isopeptidase. In CSN5, the JAMM domain is responsible for the cleavage of Nedd8 from cullins, resulting in an inability of cullin complexes to catalyze ubiquitin ligation to target proteins, such as HIF-1 α ,

for 26S-dependent degradation. This responsibility directly connects the CSN in dynamically preventing ubiquitylation of certain proteins and subsequent 26S proteasome-dependent degradation.

One of the first cytokine activities ever described, MIF is found over-expressed in a wide variety of human tumors, and MIF intratumoral expression strongly correlates with angiogenic growth factor expression, tumor vessel density, and risk of recurrence after resection (Chesney *et al.*, 1999; Hira *et al.*, 2005; Ren *et al.*, 2005; Shun *et al.*, 2005; White *et al.*, 2003). Given the importance of hypoxic adaptation in malignant disease severity and progression (Melillo, 2006), in-depth studies of intrinsic regulators of hypoxia-dependent HIF-1 α expression are critical. Here, we discuss detailed methods for analyzing hypoxia-mediated MIF expression, MIF-dependent HIF-1 α stability, and regulation of cullin-dependent E3 ubiquitin ligase complex function by MIF and CSN5 (Winner *et al.*, 2007).

2. Modulation of MIF Levels by Targeted shRNAs and Assessment of Knockdown Efficiency

RNA interference has proven to be a powerful tool for studying gene product function in cancer-related pathways (Fuchs and Borkhardt, 2007). Using the siDesign center from Dharmacon (www.dharmacon.com), short hairpin (shRNA) target sequences can be identified and double-stranded small interfering (siRNA) oligos tested for MIF assessment of knockdown efficiency. Detailed protocols for MIF-specific shRNA-mediated knockdown and assessment of expression are described next.

2.1. MIF-specific shRNA transfection

For shRNA transfections, cells are seeded in growth media containing 10% heat-inactivated fetal bovine serum, 1% gentamicin, and 1% L-glutamine (Invitrogen, Carlsbad, CA) at $\sim 3 \times 10^5$ cells (20–30% confluency) on 10-cm tissue culture dishes in 6 ml of medium. It is important to initiate shRNA transfections beginning with low cell density to ensure efficient transfection efficiencies. In our experience, higher cell densities generally result in lower shRNA transfection efficiency. After seeding, cells should be allowed to adhere overnight in a humidified incubator of 5% CO₂ at 37°. Of the three MIF-specific siRNAs we initially tested, two were found to very efficiently inhibit MIF expression, and one is described in detail here. The human MIF-targeted base sequence is 5'-CCTTCTGGTGGGGAGAAAT-3', and the derivative scrambled oligo (NS) sequence is 5'-CCTTCTGGT GGGGAGAAAT-3'. Annealed siRNA oligos (Dharmacon, Lafayette, CO) should be resuspended in siRNA buffer (20 mM KCl, 6 mM HEPES pH 7.5, 0.2 mM MgCl₂) made with RNase-free water to give a final 20-mM stock. RNA working precautions should be exercised when working with shRNAs. For transfections, dilute Oligofectamine (Invitrogen) in OPTIMEM media (Invitrogen) at a final ratio of 1:2.75. Mix by gentle pipetting, then incubate this mixture for 10 min. In another tube, dilute each siRNA oligo in 182.5 μ l of OPTIMEM for each milliliter of medium to a final concentration of 50 nM. After incubating for 10 min, add 15 μ l of the diluted Oligofectamine for each milliliter of medium to the tube containing the diluted siRNA oligo and mix by gentle vortexing. After incubating for an additional 20 min, remove the equivalent amount of media from cells that will be added, and add 200 μ l per each milliliter of medium to the cells in a drop-wise fashion. Incubate the transfected cells for 48 to 72 h at 5% CO₂ at 37°.

In order to rule out off-target effects of MIF shRNAs, studies should be performed to add-back soluble MIF to evaluate rescue of HIF- α expression. Repeated attempts by our laboratory to rescue the HIF destabilization phenotype induced by MIF knockdown by prokaryotically expressed recombinant MIF were unsuccessful. However, conditioned supernatants from cells expressing MIF can be used to rescue HIF-destabilizing effects associated with loss of MIF.

The specificity of this rescue must be tested by adding neutralizing monoclonal antibodies or small molecule MIF inhibitors. For human MIF reconstitution, conditioned supernatants from non-confluent cultures are preincubated for 1 h with a control monoclonal antibody (mAb, 50 µg/ml; R&D Systems, Minneapolis, MN), anti-MIF mAb (50 µg/ml; R&D Systems), dimethyl sulfoxide (DMSO), or the small molecule antagonist (ISO-1, 100 µM; EMD Biosciences, San Diego, CA). After preincubation with control or MIF-inhibiting substances, conditioned supernatants are added at a 1:1 ratio with fresh media and placed onto MIF shRNA-transfected cells.

Short hairpin RNA-mediated depletion of MIF or MIF^{-/-} murine embryonic fibroblasts displays markedly defective HIF-1α stabilization when challenged with hypoxia (1% O₂), anoxia (< 0.2% O₂), and inhibitors of prolyl hydroxylases (cobalt chloride, CoCl₂). In the case of shRNA-transfected cells 48 to 72 h post-transfection, media is replaced with fresh media, and different groups of plates are exposed to ambient pO₂ (normoxia), hypoxia (1% O₂), anoxia (< 0.2% O₂), and/or CoCl₂ (150 µM final concentration) for periods ranging between 4 and 16 h. Hypoxic or anoxic conditions are created by placing the cells in a Sheldon Bactron Anaerobic/Environment chamber.

2.2. Assessment of MIF knockdown and associated phenotypes by RT-PCR

Initial studies to evaluate knockdown efficiency for MIF should include a stringent evaluation of MIF messenger RNA (mRNA) levels. Quantitation polymerase chain reaction (q-PCR) is routinely used to evaluate not only knockdown efficiencies in cells transfected with shRNAs but also as a means of measuring HIF-1α-dependent MIF and vascular endothelial growth factor (VEGF) induction. For total RNA isolation, we use the RNeasy Mini Kit (Qiagen, Valencia, CA). Cell culture medium is removed 48 to 72 h post-shRNA transfection, and 600 µl of Buffer RLT containing 10 µl of beta (β)-mercaptoethanol is added to each plate. Plates are rotated for 10 min, and cell lysates are collected with a rubber policeman and transferred to a microcentrifuge tube. Samples are homogenized by passing the lysate through a 23-gauge needle (Becton Dickinson, Franklin Lakes, NJ) four to five times. Six hundred microliters of 70% ethanol is added and mixed by inversion. Seven hundred microliters of the lysate is then added to an RNeasy mini-column and placed in a 2-ml collection tube. After centrifuging for 15 s at a minimum of 10,000 rpm, the flow-through is discarded, and the rest of the lysate is added to the column. Repeat the centrifugation. Add 700 µl of Buffer RW1 to the column, repeat the centrifugation, and discard the flow-through and collection tube. To wash the column, add Buffer RPE onto the column (placed on a new collection tube) and centrifuge for 15 s at a minimum of 10,000 rpm. Add another 500 µl of Buffer RPE to the column and centrifuge for 2 min at a minimum of 10,000 rpm. Add 40 µl of RNase-free water to the column placed on a new 1.5-ml collection tube and centrifuge for 1 min at a minimum of 10,000 rpm. Determine RNA concentration by adding 5 µl of RNA to 995 µl of water in quartz cuvettes and measuring the absorbance at 260 nm and 280 nm with a Varian Cary 50 Bio ultraviolet (UV) spectrophotometer. Determine the volume needed for 1 µg of RNA, and bring the total volume up to 12.75 µl with RNase-free water.

For complementary DNA (cDNA) synthesis, make a master mix sufficient for all samples using the Omniscript RT kit (QIAGEN) containing 2 µl of RT Buffer, 2 µl of Deoxyribonucleotide triphosphates (dNTPs), 2 µl oligo (dT) (Sigma, St. Louis, MO), 0.25 µl RNase inhibitor (Promega, Madison, WI), and 1 µl of reverse transcriptase for each reaction. After pipetting up and down, centrifuge briefly to collect liquid at the bottom of the tubes. Add 7.25 µl of this master mix to sterile, RNase/DNase-free micro-centrifuge tubes followed by the addition of 12.75 µl RNA into the appropriate tubes. Mix while incubating at 37° for 1 h in an Eppendorf thermomixer.

Amplification is carried out by making a master mix of 5 μ l of 5 $\times$ Takara PCR mix (Takara Bio Inc, Otsu, Shiga, Japan), 0.3 μ M final concentration of forward and reverse primers (Invitrogen; discussed later), SYBr Green (Molecular Probes) diluted to a ratio of 1:25,000, and 15 μ l of water, to bring the volume up to 23.5 μ l for each reaction. Aliquot 23.5 μ l of the mixture into 25 μ l SmartCycler tubes (Cepheid, Sunnyvale, CA) and add 1.5 μ l of the template DNA to the appropriate tubes. The specific primer sequences used are:

- MIF: Forward 5'-AGAACCGCTCCTACAGCAAG-3'
- Reverse 5'-TAGGCGAAGGTGGAGTTGTT-3'
- VEGF: Forward 5' CAACATCACCATGCAGATTATGC 3'
- Reverse 5'-GCTTTCGTTTTTGCCCCCTTTC-3'
- β -actin: Forward 5'-CAAGGCCAACCGCGAGAAGA-3'
- Reverse 5'-GGATAGCACAGCCTGGATAG-3'
- HIF-1 α : Forward 5'-CGTTCCTTCGATCAGTTGTC-3'
- Reverse 5'-TCAGTGTTGGCAGTGGTAGT-3'

For real time analyses, we use a DNA Engine Opticon (BioRad, Hercules, CA) to perform the PCR amplification. Relative expression levels of mRNAs are determined using the delta C_T method. The $\Delta\Delta C_T$ is calculated as the difference between the normalized C_T values (ΔC_T) of the treatment and the control samples: $\Delta\Delta C_T = \Delta C_{T \text{ treatment}} - \Delta C_{T \text{ control}}$. $\Delta\Delta C_T$ is then converted to fold change by the following formula: fold change = $2^{-\Delta\Delta C_T}$.

2.3. Assessment of MIF knockdown and associated loss of HIF- α stability by Western blotting

Because the requirement for MIF in HIF- α stabilization is at the level of protein stability (Winner *et al.*, 2007), Western blotting techniques are routinely used to monitor not only MIF knockdown efficiency but also hypoxia, anoxia, or PHD inhibitor-induced HIF- α stability and associated transcriptional activities. For assessment of MIF expression in knockdown cells or in cells pulsed with hypoxia, whole cell lysates are used. After aspirating the media away from the treated and untreated cells, 250 μ l of 1 $\times$ radioimmunoprecipitation assay (RIPA) lysis buffer (Upstate, Charlottesville, VA) is added to 10-cm dishes. All lysis buffers contain 1 mM NaVO₄, 2 mM NaF, and a protease inhibitor cocktail (Roche Biochemical, Indianapolis, IN). Plates are placed on a rocker for 5 min, and then cell lysates are scraped and transferred to microcentrifuge tubes. Samples should be further homogenized by passing the samples through a 23-gauge needle 9 to 10 times. Centrifuge the samples for 3 min at 6000 rpm to pellet the insoluble material, and then pipette the supernatant into fresh microcentrifuge tubes. Add Laemmli sample buffer (BioRad) in a 1:1 ratio to the lysate. Mix, centrifuge briefly, and boil at 100° for 10 min.

For nuclear protein determination (HIF-1 α and HIF-2 α), cytoplasmic and nuclear extracts are prepared using components from the NE-PER kit (Pierce, Rockford, IL). Media should be completely aspirated off of the cells. Two hundred microliters of CER buffer is then added. Cells are scraped off of plates with a rubber policeman and transferred into micro-centrifuge tubes. After incubating on ice for 10 min, add 11 μ l of CERII, vortex for 5 s, incubate on ice 1 min, and vortex another 5 s. Centrifuge immediately in a microcentrifuge for 5 min at 13,000 rpm at 4°. Collect the cytoplasmic fraction into fresh microcentrifuge tubes and store at -80°. Resuspend the nuclear pellet in 100 μ l of NER by pipetting up and down. Vortex for 15 s, incubate on ice for 10 min, and repeat four times. Centrifuge at 13,000 rpm for 10 min at 4°. The supernatant is the nuclear lysate and should be aliquoted into another set of microcentrifuge tubes. These can be stored at -80°. Samples to be examined by Western blotting should be normalized for protein concentration by using the DC protein assay kit (BioRad). Briefly,

prepare a standard curve ranging from 2 mg/ml BSA and diluting down to 0.1 mg/ml BSA in appropriate lysis buffer. Prepare reagent A' by adding 20 μ l of reagent S for every 1 ml of reagent A. In a 96-well plate, add 5 μ l of sample or standard, 25 μ l of reagent A', and 200 μ l of reagent B. Mix and incubate the plate at room temperature for 15 min. Measure the absorbance with a Biotek plate reader at 750 nm.

Load precast 10% or 10 to 20% SDS-Polyacrylamide gels (BioRad), depending on the size of the protein to be detected. Run the gel at 120V on a BioRad Mini-PROTEAN 3 cell and transfer at 350 mA to a PVDF membrane (Millipore, Charlottesville, VA). Block membranes for 1 h in blocking solution (5% nonfat dried milk, 0.2% Tween-20, 1% Goat Serum in TBS). Add primary antibody to the blocking solution and probe for 1h. Wash three times for 5 min each with TBS + Tween-20 (TBS-T, 0.2% Tween-20), add secondary antibody at a 1:8000 dilution to blocking solution, and incubate 1 h. Wash the membrane four times for 5 min and visualize with ECL reagent (Pierce).

Antibodies used for immunoblotting include polyclonal and monoclonal antibodies directed against MIF (Santa Cruz Biotechnology, Santa Cruz, CA and R&D Systems, Minneapolis, MN, respectively), HIF-1 α (BD Transduction Laboratories, San Jose, CA for human and Novus Biologicals, Littleton, CO for mouse HIF-1 α), CSN5 (Bethyl Laboratories, Montgomery, TX), LDH-A (Abcam, Cambridge, MA), inducible nitric oxide synthase (iNOS; Santa Cruz, CA), β -actin (Sigma), Nedd8 (Boston Biochem, Cambridge, MA), VHL (BD Pharmingen), HIF-2 α (Stratagene, La Jolla, CA), and α -tubulin (Sigma).

2.4. Enzymatic analyses

Three-dimensional X-ray crystallographic studies have revealed that human MIF exists as a homotrimer and is structurally related to the bacterial enzymes 4-oxalocrotonate tautomerase and 5-carboxymethyl-2-hydroxymuconate isomerase (Sugimoto *et al.*, 1996; Sun *et al.*, 2005). MIF possesses the unusual ability to catalyze the tautomerization of the non-physiological substrates D-dopachrome and L-dopachrome methyl ester into their corresponding indole derivatives (Rosengren *et al.*, 1996). Measuring MIF enzymatic activity in cell and tissue lysates represents a semiquantitative and facile way to evaluate MIF cell or tissue concentrations of enzymatically active MIF. Cell or tissue samples are Dounce-homogenized in a phosphate-buffered saline (PBS)/protease inhibitor solution. After protein determination by standard Bradford assay (BioRad), 10, 25, 50, and 100 μ g of total protein is diluted into a final volume of 700 μ l PBS in a 24-well plate. In parallel, 0.1, 0.2, 0.3, 0.4, and 0.5 μ M recombinant MIF (rMIF) should be diluted into 700 μ l PBS in the same 24-well plate to be used as a standard curve. Just prior to setting up the assay, combine 4 mM L-3,4-dihydroxyphenylalanine methyl ester (Sigma) with 8 mM sodium periodate (Sigma) in a 3:2 ratio in order to obtain L-dopachrome methyl ester, which becomes a reddish color. This solution is stable for no more than 30 min. Add 300 μ l of L-dopachrome methyl ester to the cuvette, mix by pipette, and incubate 5 min at room temperature. Absorbances are read in a BioTek Power Wave multi-plate reader at OD₄₇₅ nm. From the rMIF standard curve, OD₄₇₅ test measurements that fall within the linear range of the standard curve are extrapolated against rMIF enzyme concentrations.

3. Analysis of MIF-Dependent CSN5 and COP9 Signalosome Function

Macrophage migration inhibitory factor-dependent stabilization of HIF-1 α induced by hypoxia or PHD inhibitors involves the functional modulation of CSN5 (Winner *et al.*, 2007). CSN5-mediated HIF stabilization is proposed to be independent of its deneddylating activity and is likely independent of the CSN holocomplex (Bemis *et al.*, 2004). In contrast, MIF was originally demonstrated to bind to and inhibit CSN5-dependent turnover of p27^{Kip1} (Kleemann *et al.*, 2000) in a process that is likely CSN-dependent. While these two findings may at first

appear paradoxical, combined they may help to explain how MIF acts to modulate CSN function. Studies have shown that, while the majority of cellular CSN5 exists in large, CSN-associated complexes, CSN5 is also found in smaller complexes or in monomeric form outside of the CSN (Kwok *et al.*, 1998; Tomoda *et al.*, 2004). It has been postulated that, depending on expression levels of CSN5, the levels of free or small complex CSN5 can act independently of the CSN (Richardson and Zundel, 2005). In the case of CSN5-dependent HIF-1 α stabilization, the net effect of CSN5 overexpression would increase small complex or monomeric CSN5 that would be accessible to HIF-1 α binding and subsequent stabilization (Bemis *et al.*, 2004). Because MIF inhibits CSN5-dependent, CSN-mediated actions while promoting CSN-independent CSN5-mediated functions (Kleemann *et al.*, 2000; Winner *et al.*, 2007), it is possible that MIF modulates CSN5 functions by promoting the levels or activity of free or small complex CSN5. Protocols for the investigation of MIF-dependent CSN5/JAB1 function are described next.

3.1. CSN5 co-immunoprecipitations

To investigate CSN5/HIF-1 α and CSN5/MIF association in normoxic or hypoxic cells, primary immunoprecipitation of CSN5 is used. Briefly, 1 $\times$ IP lysis buffer (20 mM Tris-HCL pH 8.0, 137 mM NaCl, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton X-100, 10% glycerol, and 1.5 mM MgCl₂) is added to 10-cm tissue culture dishes and rocked for 5 min. After scraping cells into lysis buffer and transferring to microcentrifuge tubes, lysates are homogenized with a 23-gauge needle and spun at 6000 rpm for 3 min. Supernatant is transferred to a new tube, and 1 μ g of the polyclonal CSN5 antibody (Bethyl Laboratories, Montgomery, TX) is added to lysates. In parallel, lysis buffer alone should be incubated with 1 μ g of CSN5 antibody to serve as an antibody background control during Western blotting. Rotate samples for 3 h to overnight at 4 $^{\circ}$ and then add 30 μ l of Protein A/G agarose beads (Santa Cruz) for 1 h at 4 $^{\circ}$. Pellet the beads by centrifuging for 30 s at 6000 rpm. Carefully aspirate supernatant and wash the pellet by adding 500 μ l of 1 $\times$ IP lysis buffer, mixing, and centrifuging for 30 s at 6000 rpm. Repeat the washing five times. To a dry pellet, add 35 μ l of 2 $\times$ Laemmli sample buffer and dissociate the proteins from the beads by boiling at 100 $^{\circ}$ for 10 min. Spin the samples briefly to pellet the beads and load the entire volume. Immunoblotting for either HIF-1 α or MIF should be performed as previously described.

3.2. CSN-dependent neddylation

The CSN serves to modulate the activities of Skp1/cullin/F-box protein (SCF)-containing ubiquitin ligase complexes by removing ubiquitin-like Nedd8 from SCF scaffolding proteins, cullins (Lyapina *et al.*, 2001). Disruption of the CSN results in enhanced cullin neddylation and the loss of SCF ubiquitin ligase activity due to increased turnover of F-box-containing proteins (Cope and Deshaies, 2006). Neddylated cullins are distinguishable by their slower mobility in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) immunoblots. Cell lysates from cells transfected with scrambled or MIF shRNA oligos are run for an extended time on 10% pre-cast polyacrylamide gels to adequately resolve the neddylated and deneddylated cullins. Immunoblotting against Cul2 (Zymed Laboratories, San Francisco, CA) will reveal distinct upper and lower bands representing neddylated and deneddylated Cul2, respectively. Cul1 and Cul4A neddylation patterns can be assessed in a similar fashion.

3.3. Determination of CSN-associated versus-disassociated CSN5

As previously discussed, a potential explanation for the differently observed activities of MIF on CSN5 may lie in the relative CSN-associated versus-disassociated CSN5 levels or activities. To determine potential MIF influences on the levels of small complex or “free” CSN5, gel filtration analysis of MIF-containing and depleted cell lysates is performed. A protocol for gel filtration of Arabidopsis Jab1/CSN5 homologs (Kwok *et al.*, 1998) has been adapted for

mammalian cell studies. Briefly, two 10-cm dishes of cells transfected with scrambled or MIF shRNA are scraped into a buffer containing 25 mM Tris (pH 8.0), 10 mM NaCl, 10 mM MgCl₂, 5 mM EDTA, 10 mM β -mercaptoethanol, and protease inhibitors. On ice, lysates should be homogenized with 15 strokes of a Dounce homogenizer followed by centrifugation at 3000 rpm for 5 min. Soluble protein extract (250 μ g) is fractionated by a Superose 6 10/300 gel filtration column fitted onto a BioRad BioLogic DuoFlow FPLC. Fractions (0.5 ml) are collected, TCA-precipitated, and analyzed by Western blotting for CSN5 and CSN8 (Bethyl Laboratories).

4. Determination of Tumor-Associated MIF Expression and MIF Polymorphic Disparity

Hypoxia is a strong physiologic regulator of MIF transcription in normal and malignant cells, and HIF-1 α has now been shown to be necessary for hypoxia-induced MIF expression (Baugh *et al.*, 2006; Welford *et al.*, 2006; Winner *et al.*, 2007). Numerous studies have additionally demonstrated that MIF is found overexpressed in many different human tumor types (Mitchell, 2004). Combined with our findings that plasma MIF levels are elevated in pancreatic cancer patients (Winner *et al.*, 2007), it is conceivable that MIF may represent a novel biomarker for intratumoral hypoxia. Additionally, because steady state levels of MIF dictate the stabilization of hypoxia-induced HIF-1 α in human cancer cells, it is likely that relative MIF and HIF levels closely correlate with each other. Studies designed to evaluate MIF and HIF expression levels from tumor biopsy sections are described next.

A polymorphism in the promoter of the *Mif* gene regulates MIF expression and both immune- and inflammatory-associated disease severity (Barton *et al.*, 2003; Baugh *et al.*, 2002; Hizawa *et al.*, 2004). It was found that a tetranucleotide CATT repeat polymorphism exists in the human MIF promoter region at position -794 (Baugh *et al.*, 2002). Identified individuals who are homozygous or heterozygous for these repeats are designated individual alleles as *Mif*5-CATT, 6-CATT, 7-CATT, or 8-CATT. Functionally, the lowest number of CATT repeats correlates with low MIF expression while increased numbers of repeats correlates with increased expression. Increased repeat polymorphic individuals have been described as having an increased risk or severity of rheumatoid arthritis (Baugh *et al.*, 2002), inflammatory polyarthritis (Barton *et al.*, 2003), juvenile idiopathic arthritis (Hizawa *et al.*, 2004), or systemic lupus erythematosus (Sanchez *et al.*, 2006). Given the importance of MIF in modulating HIF-1 α -dependent responses and tumoral hypoxic adaptation, procedures designed to determine MIF polymorphism disparity in cancer patients versus HIF expression and hypoxic adaptation are included in the following section.

4.1. Immunohistochemistry of MIF and CSN5 tumor expression levels and correlation to hypoxic adaptation

Serial human tumor sections can be analyzed for MIF, CSN5, HIF-1 α , GLUT-1 (to evaluate HIF-dependent expression), and blood vessel content by antifactor VIII using established techniques and antibodies. Antibodies reactive against dewaxed paraffin sections are as follows: MIF (R&D Systems), CSN5 (Zymed), HIF-1 α , HIF-2 α , Factor VIII, and GLUT-1 (Pharmingen). The antigen retrieval process after dewaxing follows a method adapted from Boddy *et al.* (2005). Dewaxed slides undergo pressurized steam treatment at 15 psi for 3 min. Peroxidases from slide samples are then blocked by incubating slides in 0.3% hydrogen peroxidase in 0.1% sodium azide for 10 min. After blocking and staining with the appropriate antibodies (1:50 dilutions for all), appropriate secondary antibodies are added, followed by DAB development. After counterstaining with hematoxylin, three to seven fields from each slide are examined at $\times 200$ magnification and scored on a scale of 0 to 3+. Cytoplasmic and nuclear HIF- α expression should be noted and scored independently.

4.2. MIF plasma analysis and genomic DNA extraction

For plasma MIF assessment, two independent analyses can be performed. The first utilizes patient plasma samples to determine circulating MIF levels. Plasma is tested in triplicate against normal donor controls in a commercially available human MIF enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (R&D Systems). An additional aliquot from the patient's plasma is used for the promoter polymorphism studies.

Genomic DNA can be extracted from as little as 10 to 20 μ l of patient plasma collected in sodium citrate using an Easy-DNA Kit (Invitrogen) and following instructions for Protocol #1—Small Blood Samples and Hair Follicles. Care should be taken not to collect plasma in EDTA as it will interfere with the analysis. To extract genomic DNA from paraffin, slides are first deparaffinized in xylene and ethanol and allowed to air dry overnight. Tissue is scraped from slides into RNase/DNase-free microcentrifuge tubes containing Proteinase K buffer (10 mM Tris, pH 7.4, 2 mM EDTA, and 0.5% Tween-20). Heat the tubes to 99° for 1 h and cool to room temperature. Add 10 μ l of a 2-mg/ml stock of Proteinase K (QIAGEN) and digest for 48 h at 55°.

4.3. Genotyping of MIF -173 G/C and MIF⁵⁻⁸ CATT repeats from human samples

In addition to the CATT repeat MIF promoter polymorphism, a G $\rightarrow$ C polymorphism at -173 was independently discovered and frequently links with the 7 CATT repeat polymorphism (Barton *et al.*, 2003; Sanchez *et al.*, 2006). The MIF promoter -173 polymorphism analysis was achieved using an Assay-on-Demand Allelic Discrimination Assay Kit (Applied Biosystems, Foster City, CA). The PCR contains 10 to 50 ng of genomic DNA, 2.5 μ l of TaqMan master mix (Applied Biosystems), 0.25 μ l 20 $\times$ Assay-on-Demand SNP Genotyping Assay Mix, and H₂O to a total of 5 μ l. PCR is performed using 384-well plates on an ABI thermal cycler (Applied Biosystems). The reaction conditions used are 95° for 10 min followed by 50 cycles of 92° for 15 sec and 60° for 1 min.

Genotyping of MIF⁵⁻⁸ CATT repeat alleles is performed using a fluorescence-labeled PCR primer and capillary electrophoresis. Genomic DNA (50 ng in 0.5 μ l) is amplified by PCR in a total reaction volume of 25 μ l containing 5 pmols of both forward and reverse primers: forward, 5'-TGCAGGAACCAATACCCATAGG-3'; reverse, 5'-AATGGTAACTC GGGGAC.

The reverse primer is pre-labeled with 6-carboxy-fluorocine (FAM) fluorescent dye. The PCR reaction contains 22.5 μ l of PCR Supermix (Invitrogen), 0.5 μ l each of primer, and 1 μ l of H₂O. The PCR is performed in 96-well plates on a PTC-100 Peltier Thermal Cycler (BioRad). Forty PCR cycles are carried out, each with denaturation for 30 s at 95°, primer annealing at 54° for 30 s, and extension for 60 s at 72°, and completed with a final extension at 72° for 10 min. The PCR product (anticipated size of 340–352 base pairs) is diluted 1:10 with H₂O and subjected to capillary electrophoresis followed by data analysis retrieval using Genemapper (Applied Biosystems).

5. Conclusions

The protocols provided should allow investigators to evaluate MIF functional modulation of HIF-1 α stabilization and associated tumoral hypoxic adaptation. Moreover, the combined analyses of CSN5, HIF-1 α , HIF-1 α target genes, and extra- and intracellular MIF using these methods will provide important insight into the coordinate regulation of tumoral hypoxic responses and adaptation.

References

- Barton A, Lamb R, Symmons D, Silman A, Thomson W, Worthington J, Donn R. Macrophage migration inhibitory factor (MIF) gene polymorphism is associated with susceptibility to but not severity of inflammatory polyarthritis. *Genes Immun* 2003;4:487–491. [PubMed: 14551601]
- Baugh JA, Gantier M, Li L, Byrne A, Buckley A, Donnelly SC. Dual regulation of macrophage migration inhibitory factor (MIF) expression in hypoxia by CREB and HIF-1. *Biochem Biophys Res Commun* 2006;347:895–903. [PubMed: 16854377]
- Baugh JA, Chitnis S, Donnelly SC, Monteiro J, Lin X, Plant BJ, Wolfe F, Gregersen PK, Bucala R. A functional promoter polymorphism in the macrophage migration inhibitory factor (MIF) gene associated with disease severity in rheumatoid arthritis. *Genes Immun* 2002;3:170–176. [PubMed: 12070782]
- Bemis L, Chan DA, Finkielstein CV, Qi L, Sutphin PD, Chen X, Stenmark K, Giaccia AJ, Zundel W. Distinct aerobic and hypoxic mechanisms of HIF- α regulation by CSN5. *Genes Dev* 2004;18:739–744. [PubMed: 15082527]
- Boddy JL, Fox SB, Han C, Campo L, Turley H, Kanga S, Malone PR, Harris AL. The androgen receptor is significantly associated with vascular endothelial growth factor and hypoxia sensing via hypoxia-inducible factors HIF-1 α , HIF-2 α , and the prolyl hydroxylases in human prostate cancer. *Clin Cancer Res* 2005;11:7658–7663. [PubMed: 16278385]
- Chan DA, Sutphin PD, Yen SE, Giaccia AJ. Coordinate regulation of the oxygen-dependent degradation domains of hypoxia-inducible factor 1 alpha. *Mol Cell Biol* 2005;25:6415–6426. [PubMed: 16024780]
- Chesney J, Metz C, Bacher M, Peng T, Meinhardt A, Bucala R. An essential role for macrophage migration inhibitory factor (MIF) in angiogenesis and the growth of a murine lymphoma. *Mol Med* 1999;5:181–191. [PubMed: 10404515]
- Cope GA, Deshaies RJ. Targeted silencing of Jab1/Csn5 in human cells downregulates SCF activity through reduction of F-box protein levels. *BMC Biochem* 2006;7:1. [PubMed: 16401342]
- Fuchs U, Borkhardt A. The application of siRNA technology to cancer biology discovery. *Adv Cancer Res* 2007;96:75–102. [PubMed: 17161677]
- Hira E, Ono T, Dhar DK, El Assal ON, Hishikawa Y, Yamanoi A, Nagasue N. Overexpression of macrophage migration inhibitory factor induces angiogenesis and deteriorates prognosis after radical resection for hepatocellular carcinoma. *Cancer* 2005;103:588–598. [PubMed: 15612021]
- Hizawa N, Yamaguchi E, Takahashi D, Nishihira J, Nishimura M. Functional polymorphisms in the promoter region of macrophage migration inhibitory factor and atopy. *Am J Respir Crit Care Med* 2004;169:1014–1018. [PubMed: 14962818]
- Hon WC, Wilson MI, Harlos K, Claridge TD, Schofield CJ, Pugh CW, Maxwell PH, Ratcliffe PJ, Stuart DI, Jones EY. Structural basis for the recognition of hydroxyproline in HIF-1 α by pVHL. *Nature* 2002;417:975–978. [PubMed: 12050673]
- Kim W, Kaelin WG Jr. The von Hippel-Lindau tumor suppressor protein: New insights into oxygen sensing and cancer. *Curr Opin Genet Dev* 2003;13:55–60. [PubMed: 12573436]
- Kim WY, Kaelin WG. Role of VHL gene mutation in human cancer. *J Clin Oncol* 2004;22:4991–5004. [PubMed: 15611513]
- Kleemann R, Haussler A, Geiger G, Mischke R, Burger-Kentischer A, Flieger O, Johannes FJ, Roger T, Calandra T, Kapurniotu A, Grell M, Finkelmeier D, Brunner H, Bernhagen J. Intracellular action of the cytokine MIF to modulate AP-1 activity and the cell cycle through Jab1. *Nature* 2000;408:211–216. [PubMed: 11089976]
- Kwok SF, Solano R, Tsuge T, Chamovitz DA, Ecker JR, Matsui M, Deng XW. Arabidopsis homologs of a c-Jun coactivator are present both in monomeric form and in the COP9 complex, and their abundance is differentially affected by the pleiotropic cop/det/fus mutations. *Plant Cell* 1998;10:1779–1790. [PubMed: 9811788]
- Lyapina S, Cope G, Shevchenko A, Serino G, Tsuge T, Zhou C, Wolf DA, Wei N, Shevchenko A, Deshaies RJ. Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. *Science* 2001;292:1382–1385. [PubMed: 11337588]
- Melillo G. Inhibiting hypoxia-inducible factor 1 for cancer therapy. *Mol Cancer Res* 2006;4:601–605. [PubMed: 16940159]

- Mitchell RA. Mechanisms and effectors of MIF-dependent promotion of tumourigenesis. *Cell Signal* 2004;16:13–19. [PubMed: 14607271]
- Ren Y, Law S, Huang X, Lee PY, Bacher M, Srivastava G, Wong J. Macrophage migration inhibitory factor stimulates angiogenic factor expression and correlates with differentiation and lymph node status in patients with esophageal squamous cell carcinoma. *Ann Surg* 2005;242:55–63. [PubMed: 15973102]
- Richardson KS, Zundel W. The emerging role of the COP9 signalosome in cancer. *Mol Cancer Res* 2005;3:645–653. [PubMed: 16380502]
- Rosengren E, Bucala R, Aman P, Jacobsson L, Odh G, Metz CN, Rorsman H. The immunoregulatory mediator macrophage migration inhibitory factor (MIF) catalyzes a tautomerization reaction. *Mol Med* 1996;2:143–149. [PubMed: 8900542]
- Sanchez E, Gomez LM, Lopez-Nevot MA, Gonzalez-Gay MA, Sabio JM, Ortego-Centeno N, de Ramon E, Anaya JM, Gonzalez-Escribano MF, Koeleman BP, Martin J. Evidence of association of macrophage migration inhibitory factor gene polymorphisms with systemic lupus erythematosus. *Genes Immun* 2006;7:433–436. [PubMed: 16724072]
- Shun CT, Lin JT, Huang SP, Lin MT, Wu MS. Expression of macrophage migration inhibitory factor is associated with enhanced angiogenesis and advanced stage in gastric carcinomas. *World J Gastroenterol* 2005;11:3767–3771. [PubMed: 15968736]
- Sugimoto H, Suzuki M, Nakagawa A, Tanaka I, Nishihira J. Crystal structure of macrophage migration inhibitory factor from human lymphocyte at 2.1 Å resolution. *FEBS Lett* 1996;389:145–148. [PubMed: 8766818]
- Sun B, Nishihira J, Yoshiki T, Kondo M, Sato Y, Sasaki F, Todo S. Macrophage migration inhibitory factor promotes tumor invasion and metastasis via the Rho-dependent pathway. *Clin Cancer Res* 2005;11:1050–1058. [PubMed: 15709171]
- Tomoda K, Yoneda-Kato N, Fukumoto A, Yamanaka S, Kato JY. Multiple functions of Jab1 are required for early embryonic development and growth potential in mice. *J Biol Chem* 2004;279:43013–43018. [PubMed: 15299027]
- Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic helix-loop-helix PAS heterodimer regulated by cellular O₂ tension. *Proc Natl Acad Sci USA* 1995;92:5510–5514. [PubMed: 7539918]
- Welford SM, Bedogni B, Gradin K, Poellinger L, Broome PM, Giaccia AJ. HIF-1 α delays premature senescence through the activation of MIF. *Genes Dev* 2006;20:3366–3371. [PubMed: 17142669]
- White ES, Flaherty KR, Carskadon S, Brant A, Iannettoni MD, Yee J, Orringer MB, Arenberg DA. Macrophage migration inhibitory factor and CXC chemokine expression in non-small cell lung cancer: Role in angiogenesis and prognosis. *Clin Cancer Res* 2003;9:853–860. [PubMed: 12576459]
- Winner M, Koong AC, Rendon BE, Zundel W, Mitchell RA. Amplification of tumor hypoxic responses by macrophage migration inhibitory factor-dependent hypoxia-inducible factor stabilization. *Cancer Res* 2007;67:186–193. [PubMed: 17210698]
- Wolf DA, Zhou C, Wee S. The COP9 signalosome: An assembly and maintenance platform for cullin ubiquitin ligases? *Nat Cell Biol* 2003;5:1029–1033. [PubMed: 14647295]