Release of macrophage migration inhibitory factor by neuroendocrine-differentiated LNCaP cells sustains the proliferation and survival of prostate cancer cells

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Abstract

The acquisition of neuroendocrine (NE) characteristics by prostate cancer (PCa) cells is closely related to tumour progression and hormone resistance. The mechanisms by which NE cells influence PCa growth and progression are not fully understood. Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine involved in oncogenic processes, and MIF serum levels correlate with aggressiveness of PCa. Here, we investigated the regulation and the functional consequences of MIF expression during NE transdifferentiation of PCa cells. NE differentiation (NED) of LNCaP cells, initiated either by increasing intracellular levels of cAMP or by culturing cells in an androgen-depleted medium, was associated with markedly increased MIF release. Yet, intracellular MIF protein and mRNA levels and *MIF* gene promoter activity decreased during NED of LNCaP cells, suggesting that NED favours MIF release despite decreasing MIF synthesis. Adenoviral-mediated forced MIF expression in NE-differentiated LNCaP cells increased cell proliferation without affecting the expression of NE markers. Addition of exogenous recombinant MIF to LNCaP and PC-3 cells stimulated the AKT and ERK1/2 signalling pathways, the expression of genes involved in PCa, as well as proliferation and resistance to paclitaxel and thapsigargin-induced apoptosis. Altogether, these data provide evidence that increased MIF release during NED in PCa may facilitate cancer progression or recurrence, especially following androgen deprivation. Thus, MIF could represent an attractive target for PCa therapy.

Key Words

- ▶ neuroendocrine differentiation
- ▶ macrophage migration inhibitory factor
- ▶ proliferation
- ▶ apoptosis
- ▶ prostate cancer

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Introduction

Prostate cancer (PCa) is a major oncological problem. In the western world, males have a 16% chance of developing clinically apparent PCa (Jemal *et al.* 2010), and although curative treatments are available for early disease, advanced PCa progresses to the castrate-resistant state and is fatal in the majority.

The epithelial compartment contains a minor component of neuroendocrine (NE) cells that may regulate the growth, differentiation and secretory function of the normal prostate (Sun *et al.* 2009). All adenocarcinomas of the prostate contain some NE cells, and about 10% have abundant NE cells (Abrahamsson *et al.* 1987). NE differentiation (NED) is increased in high-grade and high-stage tumours, and particularly in hormonally treated tumours and castrate-resistant tumours (Sun *et al.* 2009, Sagnak *et al.* 2011). NE tumour cells do not express the androgen receptor (AR) that may favour tumour cell survival during androgen deprivation. Secreted neuropeptides inhibit *in vitro* apoptosis, increase androgen-independent growth and invasion of PCa cells (Salido *et al.* 2004, Vilches *et al.* 2004). In human prostatic tissue, cancer cells expressing the anti-apoptotic molecule BCL2 are generally in close proximity to NE cells (Segal *et al.* 1994).

The origin of NE cells in PCa is controversial, but it is becoming increasingly clear that NE cells in primary PCa are different from NE cells in normal prostate (Sun *et al.* 2009). Furthermore, NED is more prevalent in bone metastasis lesions than within the primary tumour foci, suggesting the occurrence of a transdifferentiation process in metastatic PCa cells (Cheville *et al.* 2002). *In vitro*, LNCaP cells can acquire the NE phenotype through androgen deprivation (Burchardt *et al.* 1999) or exposure to agents increasing intracellular levels of cAMP (Cox *et al.* 1999). Using this latter model, we previously unravelled a critical role of the scaffold protein IB1/JIP-1 (mitogen-activated protein kinase 8 interacting protein 1 (MAPK8IP1)) in the control of c-Jun N-terminal kinase (JNK) activity and resistance to apoptosis in prostatic cells (Tawadros *et al.* 2005).

Intracellular phosphoinositide-3-kinase (PI3K)/AKT signalling has been shown in preclinical and clinical studies to be associated with PCa. AKT1 phosphorylation is associated with higher Gleason grade, advanced stage and poor prognosis (Ayala *et al.* 2004) and is a predictor of disease recurrence after primary treatment (Kreisberg *et al.* 2004), and associated with castrate-resistant PCa (CRPC). In addition, many prostate tumours display deregulated growth factor signalling, which may result

in the activation of ERK signalling (Gioeli 2005). The PI3K/AKT and ERK1/2 MAPK signalling pathways are alternatively and/or synchronously expressed in advanced PCa, functioning cooperatively to promote tumour growth and the emergence of castrate resistance (Uzgare & Isaacs 2004, Gao *et al.* 2006).

Macrophage migration inhibitory factor (MIF) is a mediator initially identified by its ability to halt the random migration of macrophages *in vitro*. More recently, it has been characterized as a pro-inflammatory cytokine with a key regulatory role in inflammation and immune responses (Calandra & Roger 2003, Calandra *et al.* 2003). Additionally, MIF functions in many oncogenic processes including cell proliferation, angiogenesis and suppression of host-tumour cell immune surveillance (Mitchell 2004, Bucala & Donnelly 2007). MIF is directly associated with the growth of lymphoma, melanoma, and colon cancer cells, and therapy with either anti-MIF immunoglobulins or MIF antisense oligonucleotides confers anti-tumour activity (Chesney *et al.* 1999, Meyer-Siegler *et al.* 2004). MIF induces cell proliferation via sustained activation of ERK1/2 MAPKs and promotes cell survival through the inhibition of p53 and the activation of PI3K/AKT signalling (Hudson *et al.* 1999, Mitchell *et al.* 1999, Fingerle-Rowson *et al.* 2003, Petrenko *et al.* 2003).

Several lines of evidence suggest that MIF is closely associated with PCa progression. MIF expression is increased in human metastatic PCa cells compared with normal prostate glands, and in metastatic cell lines compared with normal ones (Meyer-Siegler *et al.* 2005). MIF levels are also increased in patients with PCa compared with controls and in patients with metastasis and/or extraprostatic disease. Moreover, increased MIF levels correlate with higher clinical T stage, Gleason score and the extent of disease as a percentage of biopsy cores (Meyer-Siegler *et al.* 2005, Muramaki *et al.* 2006). Interestingly, *MIF* gene polymorphisms increasing MIF expression are associated with higher PCa incidence (Meyer-Siegler *et al.* 2007). Herein, we studied the regulation and the functional consequences of MIF expression during NE transdifferentiation of LNCaP cells.

Material and methods

Cell lines and reagents

LNCaP cells, PC-3 and HeLa cells were purchased from ATCC (Rockville, MD, USA). All cell lines were cultured in

RPMI-1640 medium (Invitrogen) supplemented with 12% FBS, 110 U/ml penicillin and 110 µg/ml streptomycin. LNCaP cells were cultured at low passage number (<22) and were NE differentiated using 3-isobutyl-methylxanthine (IBMX; Sigma–Aldrich) diluted in dimethyl sulphoxide (DMSO) as described previously (Tawadros *et al.* 2005), and using an androgen-depleted medium as reported (Burchardt *et al.* 1999). For culturing in serum-reduced (SR) conditions, LNCaP cells were allowed to adhere to plastic 48 h in normal medium. The medium was then replaced by RPMI-1640 medium supplemented with 5% charcoal/dextran-treated FBS (containing <0.1 nM testosterone; Hyclone, Logan, UT, USA) and 1% glutamine. LNCaP cells were transduced with empty control and MIF-expressing adenoviruses (Roger *et al.* 2003, Rendon *et al.* 2007). Five hours after transduction, the medium was changed and cells were incubated with DMSO or IBMX for 48 h or in SR medium for 7 days. The MIF small-molecule inhibitor (*S,R*)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) was obtained from Merck Millipore (Darmstadt, Germany). Control and anti-MIF antibodies were obtained as described previously (Roger *et al.* 2003).

MIF ELISA

For NED induced by IBMX, medium was replaced every two days by a fresh medium containing IBMX. For NED induced by culture in androgen-depleted medium, medium was replaced 24 h before collection with a fresh medium. Conditioned media were centrifuged for 5 min at 200 g, filtered (0.22 µm) and used to quantify MIF concentrations by ELISA (Delaloye *et al.* 2012, Renner *et al.* 2012). Cells were recovered and enumerated to normalize MIF concentrations to cell number. For each time point, a control was performed with cells cultured in a normal medium. MIF was undetectable in a fresh medium.

MTT assay

LNCaP and PC-3 cells were seeded at 8000 cells/well in 96 wells and cultured as mentioned in the legends of Figures 1B, 3D, 3E and 5A, B, E and F. Twenty microlitres of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml in PBS) were added to the medium. After 2 h, the medium was removed, plates were dried and DMSO was added to solubilize formazan crystals. Optical density was read at 590 and 670 nm (background value). Each batch was tested in triplicate. Cell proliferation was also monitored by measuring [³H]thymidine incorporation over 18 h, as

described previously (Roger *et al.* 2011), which gave results similar to the MTT assay (data not shown).

Apoptosis

LNCaP and PC-3 cells, grown on coverslips and serum starved, were incubated with recombinant MIF for 6 h before the addition of paclitaxel (Sigma–Aldrich T7191) and thapsigargin (Sigma–Aldrich T9033). Evaluation of the apoptotic rate was based on the analysis of 600 cells stained with Hoechst dye (Tawadros *et al.* 2005).

RNA isolation and analysis by northern blot and quantitative real-time-PCR

Total RNA was isolated from cells homogenized in TriPure Isolation Reagent (Roche Applied Sciences, Rotkreuz, Switzerland). Northern blots were performed and hybridized with human MIF and β-actin probes (Roger *et al.* 1998b, Tawadros *et al.* 2002). One microgram of DNase-treated (DNA-free kit; Ambion, Cambridge, UK) RNA was reverse-transcribed using the ImProm-II RT System (Promega). Quantitative real-time (RT)-PCR was performed using a LightCycler Instrument (Roche Applied Sciences) and the QuantiTect SYBR Green PCR Kit (Qiagen). Reaction mixtures (20 µl) contained 4 µl of cDNA, 10 µl of 2×PCR Master Mix and 3 pmol of each primer. Amplifications consisted of one cycle of 15 min at 95 °C followed by 45 cycles of 15 s at 95 °C, 20 s at 50 °C and 15 s at 72 °C. Analysis of data was performed using LightCycler Software version 3.5 (Roche). PCR efficiency was calculated by analysing the amplification of serial dilutions of the target gene that served as a reference standard. The following primers (sense and antisense, all 5′–3′) were used in RT-PCR: *MAPK8IP1* (also known as *IB1/JIP-1*), TCAGTCCAGG-TTCCCTATCAC and TTGACGCCTATCTTCACACC; *SYN* (synaptophysin), CCAACAAGACCGAGAGTG and ATGG-AGTAGAGGAAGGCAAA; *PSA* (kallikrein-related peptidase 3 (*KLK3*)), ATGTGGGTCCCGGTTGTCTT and TCCACA-ATCCGAGACAGGA; *enolase 2* (gamma, neuronal; *ENO*), AGCTGAGGGATGGAGACAAA and CTTACACACGGCCA-GAGACA; *MIF*, CAGAACCGCTCCTACAGCAA and GAG-TTGTTCAGCCACACA; *cyclin E2* (*CCNE2*), TATGACA-CCACCGAAGAGCA and TAGGGCAATCAATCACAGCA; *B-cell CLL/lymphoma 2* (*BCL2*), GCATTTCACGTCAACAGAA and AACAGGCCACGTAAAGCAAC; *jun* proto-oncogene (*JUN*), TCCTATGACGATGCCCTCAAC and GTGTTCTGG-CTGTGCAGTTC; *AR*, GGCTACACTCGGCCCTCA and AGGCAGGTCTTCTGGGGTGGA; vascular endothelial growth factor A (*VEGFA*), CGAAACCATGAACTTCTGC and

CCTCAGTGGGCACACACTCC; CD74, CTGCCCAATCTC-CATCTGTC and GTGCCTTCCCATCTCGTC; 18S, CTCAA-CACGGGAAACCTCAC and AGACAAATCGCTCCACCAAC.

Western blot analyses

LNCaP and PC-3 cells were lysed either in Passive Lysis Buffer (Promega) or in SDS buffer (62.5 mM Tris-EDTA, pH 6.8, 5% SDS; used for detection of phosphorylated and total AKT and ERK1/2). Protein extracts were heated at 95 °C in loading buffer, fractionated by electrophoresis in 15% polyacrylamide gels and immunoblotted overnight onto Immobilon PVDF membranes (Millipore, Billerica, MA, USA). Membranes were incubated for 1 h at room temperature in PBS containing 5% milk and 0.1% Tween-20 (blocking buffer) before overnight incubation at 4 °C with antibodies specific for ENO (Dakocytomation, Baar, Switzerland), MIF (purified from the sera of MIF-immunized New Zealand white rabbits), tubulin (Sigma-Aldrich), phospho-AKT (S473), AKT, phospho-ERK1/2 (Thr202/Tyr204) and ERK1/2 (cat no. 4051, 9272, 9101, 4695 respectively; Cell Signaling Technology, Danvers, MA, USA). Antigen-antibody complexes were detected with HRP-conjugated secondary antibodies and the ECL Western Blotting Analysis System (GE Healthcare, Uppsala, Sweden).

Plasmid construction, transient transfection and luciferase assays

Human MIF promoter constructs were reported previously (Roger *et al.* 2007). LNCaP and HeLa cells were transfected with luciferase and pRLTK *Renilla* luciferase vectors as described previously (Roger *et al.* 1998a). Luciferase activity was normalized to *Renilla* luciferase activity. All experiments were repeated at least three times in duplicate.

Statistical analysis

Densitometric analyses were performed using a Molecular Dynamics Scanner (Sunnyvale, CA, USA). Data are expressed as mean $\pm$ S.E.M. Comparisons between groups were assessed using Student's *t*-test. Statistical significance was defined at a value of * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Results

Increased MIF release by NE-differentiated LNCaP cells

The NED of LNCaP cells was induced by increasing intracellular cAMP levels using IBMX (Tawadros *et al.*

2005). Four days of treatment with IBMX increased the expression of the NE marker neuron-specific enolase (NSE, official name ENO; Cox *et al.* 1999) and decreased cell proliferation in a dose-dependent manner (Fig. 1A and B). The NED of LNCaP cells was also achieved by growing cells in an androgen-depleted environment (i.e. in SR medium) mimicking clinical androgen ablation therapy (Burchardt *et al.* 1999). The expression of the NE-associated transcripts *SYP* (synaptophysin) and *IB1/JIP-1* was significantly increased in LNCaP cells cultured for 1 month in SR medium, whereas that of the prostatic epithelial marker *PSA* was significantly decreased (Fig. 1C).

The amount of MIF released by LNCaP cells over 48 h during NED was quantified by ELISA. IBMX increased the release of MIF by LNCaP cells, rising three- to fivefold using 1–1.5 mM IBMX for 4–6 days ($P < 0.001$; Fig. 1D). In line with this observation, MIF release was also significantly augmented 18 days after starting SR-induced NED of LNCaP cells (1.5-fold, $P < 0.05$), gradually increasing to a plateau at day 30 (fivefold, $P < 0.01$) and remaining elevated for > 2 months (Fig. 1E).

Reduced MIF synthesis during NED of LNCaP cells

To assess whether increased release of MIF during NED resulted from increased MIF synthesis, intracellular MIF was quantified by western blot and *MIF* mRNA by northern blot in LNCaP cells cultured for 0, 2, 4 and 6 days with 0, 0.5, 1 and 1.5 mM IBMX. Unexpectedly, IBMX reduced both intracellular MIF protein and *MIF* mRNA levels (3.3-fold reduction using 1.5 mM IBMX for 6 days, $P < 0.001$; Fig. 2A). Similarly, LNCaP cells cultured in SR media for 1 or 2 months decreased intracellular MIF protein and *MIF* mRNA levels ($P < 0.001$) approximately threefold (Fig. 2B). *In vitro*, NED is transient and cells can fully revert to their original phenotype in the absence of inducers (Cox *et al.* 1999). Interestingly, 2 days after the withdrawal of IBMX from the culture medium of NE-differentiated LNCaP cells, *MIF* mRNA expression levels increased again 1.6-fold (Fig. 2C).

To verify that *MIF* gene activity was reduced during NED of LNCaP, luciferase activity was measured in LNCaP cells transiently transfected with a MIF promoter luciferase reporter vector (MIF promoter ranging from –1072 to +129; Roger *et al.* 2007) and cultured for 8, 24 and 48 h with 0, 0.5 and 1.5 mM IBMX (Fig. 2D). Consistent with a reduced *MIF* mRNA expression, MIF promoter activity was repressed in a time- and dose-dependent manner by IBMX (3.5-fold repression with 1.5 mM IBMX for 48 h, $P < 0.01$).

Figure 1

MIF release increased during NE differentiation of LNCaP cells. (A) Western blot analysis of neuron-specific enolase (NSE) and tubulin expression in LNCaP cells cultured for 4 days with increasing concentrations of IBMX (0, 0.25, 0.5, 1 and 1.5 mM) or DMSO (D). (B) MTT assay of LNCaP cells treated with IBMX for 4 days. Data are means $\pm$ S.E.M. of triplicate samples from two independent experiments. * P < 0.05, ** P < 0.01 and *** P < 0.001 vs no IBMX. (C) Quantitative RT-PCR analysis of synaptophysin (*SYP*), prostate specific antigen (*PSA*) and *IB1/JIP-1* mRNA expression in LNCaP cells cultured during 1 month in normal (control) or serum-reduced (SR) medium. *SYP*, *PSA* and *IB1/JIP-1* mRNA levels were normalized to *18S* mRNA levels and expressed relative to the ratio obtained in control cells set at 1. Data are means $\pm$ S.E.M. of duplicate samples from two independent

experiments. *** P < 0.001 vs control medium. (D) MIF release by LNCaP cells cultured for 0.3, 2, 4 and 6 days with increasing concentrations of IBMX (0, 0.5, 1 and 1.5 mM). Medium was changed every two days. MIF concentrations were normalized to cell number, and to MIF release by cells cultured with DMSO (set at 1). Data are means $\pm$ S.E.M. of duplicate samples from three independent experiments. * P < 0.05, and *** P < 0.001 vs no IBMX. (E) MIF release by LNCaP cells cultured in SR medium. Values represent MIF release during 24 h. Concentrations were normalized to cell number, and to MIF release by cells cultured in control medium (set at 1). Data are means $\pm$ S.E.M. of duplicate samples from three independent experiments. * P < 0.05 and ** P < 0.01 vs control medium.

In contrast, MIF promoter-driven luciferase activity was increased in HeLa cells cultured for 48 h with 0.5 and 1.5 mM IBMX (P < 0.001 vs no IBMX; Fig. 2D). Collectively, these data indicated that NED of LNCaP cells was associated with reduced *MIF* gene expression and MIF intracellular cell content, concomitantly with increased MIF release.

Adenoviral-derived MIF overexpression sustains proliferation during NED

To test whether increased MIF release during NED could modulate MIF own expression, LNCaP cells were cultured with increasing concentrations of recombinant MIF, and *MIF* mRNA levels were quantified by RT-PCR (Fig. 3A). These experiments revealed that recombinant MIF did not modulate *MIF* mRNA expression. We then overexpressed MIF in LNCaP cells using an adenoviral gene transfer approach (Lugrin *et al.* 2009). Western blot analysis of

LNCaP cells transduced with an empty adenovirus control (Ad-Ctrl) and MIF-expressing adenovirus (Ad-sMIF) showed that Ad-sMIF increased MIF expression strongly (Fig. 3B). NSE expression similarly increased in Ad-Ctrl- and Ad-sMIF-transduced LNCaP cells treated for 48 h with IBMX, compared with Ad-Ctrl-transduced LNCaP cells treated with DMSO only. In the same way, *NSE*, *PSA* and *SYP* mRNA levels were comparable in Ad-Ctrl and Ad-sMIF LNCaP cells cultured in SR medium for 7 days (Fig. 3C). By contrast, proliferation in Ad-sMIF LNCaP cells was increased by 2.8-fold compared with Ad-Ctrl LNCaP cells (P < 0.05; Fig. 3D). These results suggest that MIF did not regulate the expression of NE markers, but promoted proliferation during NED of LNCaP cells. To analyse the role of extracellular MIF in that process, we measured the proliferation of LNCaP cells cultured for 6 days in SR media, complemented with recombinant MIF (25 ng/ml) with or without the MIF small-molecule inhibitor ISO-1 (10 and 100 μ M) and anti-MIF IgGs (4 mg/ml) for the last two days

Figure 2

MIF production decreased during NE differentiation of LNCaP cells. LNCaP cells were cultured with IBMX (A) or in serum-reduced (SR) medium (B) as indicated. MIF protein and *MIF* mRNA expression were quantified by western blotting (A and B, upper panels), northern blotting (A, lower panel) and RT-PCR (B, lower panel). Values are means $\pm$ S.E.M. of duplicate samples from two independent experiments. * P < 0.05 and ** P < 0.01 vs control. (C) LNCaP cells were cultured for 4 days with IBMX (1.5 mM). IBMX was withdrawn and the culture prolonged for two additional days. *MIF* mRNA expression was assessed by northern blotting after 4 and 6 days of culture and normalized to 28S/18S signals. The values represent the

average from two independent experiments performed in duplicate. * P < 0.05, ** P < 0.01. (D) LNCaP and HeLa cells were transfected with –1072/+129 human MIF promoter luciferase reporter plasmid together with a *Renilla* pRLTK vector. Cells were cultured for 8, 24 and 48 h in the presence of 0, 0.5 and 1.5 mM IBMX. Results are expressed as the ratio of luciferase activity to *Renilla* luciferase activity normalized to the value obtained in cells cultured without IBMX set at 1. Data are means $\pm$ S.E.M. of duplicate samples from three independent experiments. * P < 0.05, ** P < 0.01 and *** P < 0.001 vs no IBMX. No luciferase activity was measured in cells transfected with the empty pGL3 plasmid (not shown).

of culture. As shown in Fig. 3E, MIF increased the proliferation of LNCaP cells in SR media. In favour of an important role of extracellular MIF, this effect was blocked by ISO-1, which disrupts MIF–CD74 interaction, and by anti-MIF antibodies, which block extracellular MIF.

MIF sustains AKT and ERK1/2 signalling pathways, and the expression of markers involved in proliferation, survival and metastasis

NE cell differentiation is probably linked to PCa progression (Jin *et al.* 2004, Sagnak *et al.* 2011). Moreover, MIF has been shown to activate the AKT and ERK1/2 pathways in several cell types (Mitchell *et al.* 1999, Lue *et al.* 2007). The impact of recombinant MIF on the phosphorylation of AKT and ERK1/2 in LNCaP cells and in the more aggressive, hormone-independent PC-3 cells was therefore investigated.

In a first set of experiments, serum-starved LNCaP and PC-3 cells were cultured for 6 h with increasing

concentrations of MIF before analysing AKT and ERK1/2 phosphorylation by western blotting. These analyses revealed that AKT and ERK1/2 phosphorylation was maximally induced with 25 and 15 ng/ml MIF in LNCaP and PC-3 cells respectively (Fig. 4A and B). The effect of these MIF concentrations was tested subsequently in a kinetic study (0, 0.5, 2, 6, 12, 24 and 48 h), which showed that recombinant MIF induced a rapid (0.5 h for AKT and ERK1/2 in LNCaP cells and ERK1/2 in PC-3 cells; 2 h for AKT in PC-3 cells) and sustained (up to 24 h) phosphorylation of AKT and ERK1/2 (Fig. 4C and D). In keeping with increased intracellular signalling induced by MIF, RT-PCR revealed that recombinant MIF increased in LNCaP and PC-3 cells the expression of genes known to be involved in proliferation, survival and metastasis in PCa, including *JUN*, *CCNE2*, *BCL2*, *CD74* (which encodes a bona fide MIF receptor) and *VEGF* (Fig. 4E and F). Notably, MIF increased *AR* expression in LNCaP cells but not in PC-3 cells that do not express *AR* (Fig. 4E, data not shown).

MIF increases proliferation and apoptotic resistance of prostatic cell lines

NE cancer cells can stimulate the proliferation of adjacent non-NE cancer cells through the release of growth factors. To test whether MIF acts in this growth factor-like manner, the effect of recombinant MIF on the growth of LNCaP and PC-3 cells was tested. Proliferation was evaluated by MTT and thymidine incorporation assays, which showed similar results (data not shown). Increasing concentrations of recombinant MIF were added to serum-starved LNCaP and PC-3 cells and proliferation was monitored 48 h later (Fig. 5A and B). MIF induced a typical bell-shaped dose-response curve. Maximum proliferation of LNCaP cells (2.5-fold) was achieved using 25 ng/ml MIF, whereas that of PC-3 cells (1.8-fold) was obtained using 15 ng/ml MIF ($P < 0.01$), which correspond to the optimal concentrations activating AKT and ERK1/2 signalling (Fig. 4A and B).

To connect MIF-induced signalling and proliferation, the effect of inhibitors of the PI3K/AKT (LY294002) and ERK1/2 (PD98059) pathways on the proliferation of LNCaP and PC-3 cells was analysed. The efficiency of the two inhibitors was confirmed: LY294002 and PD98059 fully abrogated MIF-induced phosphorylation of AKT and ERK1/2 in LNCaP and PC-3 cells (Fig. 5C and D). Interestingly, inhibition of the PI3K/AKT pathway significantly decreased the proliferation of LNCaP (1.7- to 3.4-fold, $P < 0.05$) and PC-3 cells (twofold, $P < 0.05$), whereas inhibition of the ERK1/2 pathway decreased

Figure 3

Adenoviral-mediated MIF overexpression increased the proliferation of LNCaP cells. (A) Quantitative RT-PCR analysis of *MIF* mRNA expression in LNCaP cells cultured for 12 and 36 h with 0, 5, 25, 50 and 75 ng/ml recombinant human MIF. Data are means $\pm$ S.E.M. of duplicate samples from two independent experiments. (B, C and D) LNCaP cells were transduced (at day 4 in C and D) with MIF-expressing (Ad-sMIF) and empty control (Ad-Ctrl) adenoviruses. (B) Western blot analysis of MIF, NSE and tubulin expression in LNCaP cells treated for 48 h with 1 mM IBMX. (C) RT-PCR analysis of *PSA*, *NSE* and *SYP* mRNA levels in LNCaP cells cultured for 7 days in serum-reduced (SR) medium. (D) MTT assay in LNCaP cells cultured in SR media during 1 week. Data are means $\pm$ S.E.M. of duplicate samples from three independent experiments. * $P < 0.05$ vs Ad-Ctrl-infected cells. (E) MTT assay in LNCaP cells cultured in SR media during 6 days. Recombinant MIF (25 ng/ml) was added after 4 days in the presence or absence of ISO-1 (10 and 100 μ M), DMSO, control IgGs or anti-MIF IgGs (4 mg/ml). Data are means $\pm$ S.E.M. of triplicate samples from two independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs MIF.

Figure 4

Recombinant MIF activated the AKT and ERK1/2 signalling pathways and increased the expression of markers involved in proliferation, angiogenesis and survival. (A, B, C and D) Western blot analysis of total and phosphorylated (p-) AKT and ERK1/2 expression in LNCaP (A and C) and PC-3 (B and D) cells incubated for 6 h with increasing concentrations of recombinant MIF (A and B) or for 0, 0.5, 2, 6, 12, 24 and 48 h with 25 ng/ml (LNCaP cells, C) and 15 ng/ml (PC-3 cells, D) recombinant MIF. Data are means

of at least duplicate samples from two independent experiments. RT-PCR analysis of *JUN*, cyclin E2, *BCL2*, *CD74*, *AR* and *VEGF* mRNA expression in LNCaP cells (E) and PC-3 cells (F) cultured for 24 h with or without MIF. Results are expressed as described in Fig. 1C. Data are means $\pm$ s.e.m. of duplicate samples from three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs no MIF.

the proliferation of LNCaP cells (twofold, $P < 0.05$) but not of PC-3 cells (Fig. 5E and F).

In light of the results, we questioned whether MIF could confer resistance to apoptosis induced by chemotherapeutic agents. We selected for these studies paclitaxel and thapsigargin that induce apoptosis by microtubule stabilization and by inhibiting calcium transfer to endoplasmic reticulum. Apoptosis was assessed by direct microscopic evaluation of LNCaP and PC-3 cells treated with or without paclitaxel (50 nM for 48 h),

thapsigargin (20 nM for 36 h) and MIF (5–50 ng/ml), and labelled with the DNA-binding dye Hoechst 33342. Paclitaxel induced apoptosis in 40% of LNCaP cells and 30% of PC-3 cells, whereas thapsigargin induced apoptosis in 20 and 15% of the cells. Recombinant MIF significantly decreased paclitaxel-induced apoptosis in LNCaP cells (2.5-fold, $P < 0.05$) and PC-3 cells (1.7-fold, $P < 0.05$) (Fig. 6A and B). It also reduced thapsigargin-induced apoptosis in LNCaP cells (1.9-fold, $P < 0.05$), but not in PC-3 cells (Fig. 6C and D).

Figure 5

Recombinant MIF sustained the proliferation of LNCaP and PC-3 cells. Proliferation of LNCaP (A) and PC-3 (B) cells cultured for 2 days with increasing concentrations of recombinant MIF. Cells were serum starved for 16 h before the addition of recombinant MIF. Proliferation was evaluated using the MTT assay. Data are means $\pm$ S.E.M. of triplicate samples from two independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs no MIF. (C and D) Western blot analysis of total and phosphorylated (p-) AKT and ERK1/2 expression in LNCaP and PC-3 cells treated with (+) or without (-)

LY294002 (20 μ M, D) and PD98059 (30 μ M, C) 30 min prior to the addition of recombinant MIF for 6 and 24 h. Data are representative of two independent experiments. LNCaP cells (E) and PC-3 cells (F) were treated with (+) or without (-) LY294002 and PD98059 30 min prior to the addition of recombinant MIF. Proliferation was evaluated 2 days later using the MTT assay. Values obtained for cells treated without inhibitors were set at 1. Data are means $\pm$ S.E.M. of triplicate samples from two independent experiments. * $P < 0.05$ and ** $P < 0.005$ vs no inhibitor.

Discussion

In developed western countries, a significant number of men present with locally advanced or metastatic disease and many will die from this regardless of the use of state-of-the-art treatment (Zelevsky *et al.* 2011). Although androgen deprivation therapy is valuable and widely used, most patients will experience disease progression due to the development of castration resistance (Zelevsky *et al.* 2011). Despite the development of new systemic therapies, the overall survival of patients with castration resistance is < 3 years (Berthold *et al.* 2008). Castrate resistance mechanisms are complex and influenced by multiple endogenous and exogenous signals encompassing NED. Indeed, a high percentage of NE-differentiated cells are present in prostatic tissue and high concentrations of NE markers are measurable in the blood in castrate-resistant states (Sagnak *et al.* 2011). NE-differentiated cells secrete somatostatin,

5-hydroxytryptamine (serotonin), bombesin, calcitonin and parathyroid hormone-related protein, which have all been associated with PCa progression (Sagnak *et al.* 2011).

MIF values in the blood are increased in patients with PCa and have been associated with the extent of disease (Meyer-Siegler *et al.* 2005, Muramaki *et al.* 2006). To evaluate the relationship between NED and MIF levels, we used *in vitro* models of PCa, observing in time-course experiments that NED of LNCaP cells was associated with increased MIF release. This increase was durable, as seen in the SR medium-induced NE-differentiated LNCaP cells, where MIF release was sustained for at least 2 months.

MIF lacks a signal sequence and is secreted by an unconventional route that may require caspase-1 activity (Keller *et al.* 2008). Glyburide, which inhibits ATP-binding cassette transporter-dependent secretion, but not inhibitors of the endoplasmic reticulum/Golgi transport, impairs lipopolysaccharide (LPS)-stimulated MIF secretion from

Figure 6

Recombinant MIF increased resistance to paclitaxel and thapsigargin-induced apoptosis. Apoptosis of LNCaP cells (A and B) and PC-3 cells (C and D) cultured with (+) or without (-) paclitaxel (Px, 50 nM for 48 h), thapsigargin (TG, 20 nM for 36 h) and recombinant MIF. Cell apoptosis was

evaluated by Hoechst staining. Data are means $\pm$ S.E.M. of duplicate samples and are representative of three independent experiments. * $P < 0.05$ vs Px or TG without MIF.

monocytes/macrophages (Flieger *et al.* 2003). Depletion of the Golgi-associated protein p115 reduces stimulated MIF secretion. p115 may be essential for the transport of MIF from the perinuclear ring to the plasma membrane and then out of the cell (Merk *et al.* 2009). To which extent each of these mechanisms is involved in the control of MIF release during NED will be addressed in future studies.

Paradoxically, *MIF* gene expression and intracellular pools of MIF decreased during NED. The discrepancy between MIF release and MIF synthesis and storage in LNCaP cells can potentially be reconciled by the knowledge that the quantity of MIF released represents about 1% of the endogenous MIF stocks (data not shown). Of note, a low dose of IBMX (0.5 mM) did not decrease intracellular MIF, but still slightly increased MIF release and proliferation rate by NE-differentiated LNCaP cells. This suggests that the regulation of MIF production may vary according to the strength of the signal used to induce NED. Notwithstanding this, MIF content in SR medium-induced NE-differentiated LNCaP cells remained unchanged 2 months after the initial stimulus, suggesting that once the NE phenotype is acquired, a relatively stable state is reached, with concomitant and stable MIF production and MIF release.

Decreased MIF production during LNCaP NED is a dynamic process resulting from reduced *MIF* gene transcriptional activity, as deduced from the fact that MIF promoter activity decreased in IBMX-treated LNCaP cells but not in HeLa cells. Additional work will be required to fully decipher the mechanisms controlling *MIF* gene activity during NED. Possible negative regulators of *MIF* transcription are HMG box-containing protein 1 (HBP1) and natriuretic peptide receptor A, the receptor for the cardiac hormone atrial natriuretic peptide (Chen *et al.* 2010, Wang *et al.* 2011). Of note, addition of recombinant MIF in culture medium did not modify *MIF* mRNA expression (Fig. 3A), suggesting that MIF production in LNCaP cells is not responsive to extracellular levels of MIF.

The results presented herein are from cells cultured *in vitro*, and to establish the veracity of the hypothesis that MIF and NED are fundamentally related *in vivo*, it will be necessary to validate this work in animal models and humans. The data presented in this study provide a rational basis for this approach. Moreover, it is known that MIF is overexpressed in various cancers including PCa, and closely associated with tumour aggressiveness and metastasis (Meyer-Siegler *et al.* 2005, Rendon *et al.* 2009).

NED is characterized by an important decrease in proliferation concomitant with an increased expression of specific NE markers. Adenoviral-induced overexpression of MIF increased the proliferation of LNCaP cells, in agreement with the notion that MIF sustains cell proliferation (Mitchell *et al.* 1999). Yet, overexpressed MIF did not modulate the expression of NE markers, suggesting a partitioning between the proliferation and acquisition of NE markers by MIF.

MIF increased the proliferation and resistance to apoptosis in androgen-dependent LNCaP cells and, to a lesser extent, in the bone-derived androgen-independent PC-3 cells. These data suggest that MIF could be more critical in the androgen-dependent state. Considering that NE secretions are sufficient to sustain LNCaP cell growth and androgen-regulated gene expression in castrated mice (Jin *et al.* 2004), we may speculate that MIF could play a role in PCa progression following castration.

Resistance to apoptosis induced by the taxane-based cytotoxic was increased by MIF. This may have clinical implications: docetaxel, another taxane, is widely used as an active treatment for CRPC (Berthold *et al.* 2008) but resistance develops and only a proportion of patients will respond to it. We used paclitaxel in LNCaP and PC-3 cells to examine whether MIF increased post-taxane survival. The results show that apoptosis was inhibited, and it is possible to speculate that MIF may have a role in resistance to taxanes in clinical practice. This hypothesis would require validation in further studies.

MIF activated the pro-proliferative and pro-survival PI3K/AKT and ERK1/2 pathways in LNCaP and PC-3 cells. This was reflected in the gene profile results, where MIF increased the expression of genes involved in PCa development and progression such as *JUN*, *BCL2*, *AR*, *VEGF* and *CD74*. Up-regulation of *JUN* occurs in human advanced PCa and is correlated with ERK1/2 MAPK pathway activation and disease recurrence (Ouyang *et al.* 2008). *CCNE2*, which is associated with the G(1)/S-phase cell cycle progression, is increased in human metastatic PCa tissues (Wu *et al.* 2009). Interestingly, MIF was previously shown to up-regulate cyclin D1 that is also involved in the G(1)/S-phase transition (Liao *et al.* 2003). In apoptosis, overexpression of *BCL2* occurs mainly in poorly differentiated tumours and advanced stages of PCa, and is considered a marker of poor prognostic and resistance to treatment (Quinn *et al.* 2005). AR overexpression increased local intracrine synthesis of androgens, and up-regulated tyrosine kinase pathways (Chen *et al.* 2008). AR activated by androgens initiates the transcription of genes modulating the growth and

differentiation of prostate epithelial cells. Studies have shown that CRPC continues to depend on AR signalling that is reactivated despite low serum androgen levels (Chen *et al.* 2008). Finally, there were also effects on the VEGF axis. VEGF is highly expressed by PCa, and VEGF expression levels correlate with grade, vascularity and tumorigenicity of PCa (Ferrer *et al.* 1999). In line with these observations, VEGF levels correlated with MIF levels in glioblastoma (Munaut *et al.* 2002), and MIF induced a dose-dependent increase of VEGF in neuroblastoma (Ren *et al.* 2004).

Increased expression of the MIF receptor CD74 has been reported in perineural invasion in PCa (Fromont *et al.* 2012), a factor known to be associated with adverse prognosis. The fact that exogenous MIF stimulates CD74 expression in LNCaP cells raises the possibility that increased release of MIF by NE-differentiated PCa cells may facilitate CD74-dependent MIF uptake by neighbouring cells. It is also interesting to mention that CXCR4 may form with CD74 a functional MIF receptor complex, mediating MIF-stimulated, CD74-dependent AKT activation (Schwartz *et al.* 2009). Interestingly, human prostate tissues, primary cultures of prostate epithelial cells from adenocarcinomas and PCa cell lines express membrane-bound CXCR4 (Vaday *et al.* 2004). Moreover, inhibition of CXCR4 reduced aggressiveness and chemosensitized PCa cells *in vitro* and *in vivo* (Dessein *et al.* 2010, Domanska *et al.* 2012). Thus, combination therapies targeting the CD74/CXCR4/MIF axis may be valuable to interfere with cancer development.

In summary, the data presented herein are consistent with a significant role for endogenous and secreted MIF in the development of NED and tumour evolution in PCa. Interruption of MIF bioavailability or bioactivity may therefore be a legitimate therapeutic option for the treatment of high risk and metastatic PCa.

Declaration of interest

T Calandra has received research funding from and has been a consultant for Baxter AG (Vienna). All the other authors declare that there is no conflict of interest.

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Author contribution statement

T Tawadros, J-A Haefliger and T Roger designed the study and prepared the manuscript with contributions from P Jichlinski, N Clarke and T Calandra, who contributed to the experimental design, manuscript editing and review. T Tawadros, F Alonso, J-A Haefliger and T Roger performed and contributed to the analysis of the experiments.

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