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Small Interfering RNAs Induce Macrophage Migration Inhibitory Factor Production and Proliferation in Breast Cancer Cells via a Double-Stranded RNA-Dependent Protein Kinase-Dependent Mechanism

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Small interfering RNAs (siRNAs) represent a novel tool to induce gene silencing in mammalian cells and clinical trials are currently ongoing to assess the therapeutic efficacy of siRNAs in various human diseases, including age-related macular degeneration and respiratory syncytial virus infection. However, previously reported off-target, nonspecific effects of siRNAs, including activation of type I IFNs and proinflammatory cytokines, remain an outstanding concern regarding use of these agents in vivo. Macrophage-migration inhibitory factor (MIF) is a pleiotropic cytokine with well-described roles in cell proliferation, tumorigenesis, and angiogenesis and represents a target gene for siRNA-based therapy in the treatment of breast cancer. However, in this study we describe an increase in MIF production from mammary adenocarcinoma (MCF-7) cells following transfection with MIF siRNA and various control siRNAs. This effect was shown to be dose-dependent and was attenuated in the presence of a double-stranded RNA-dependent protein kinase inhibitor, 2-aminopurine. Furthermore, treatment of MCF-7 cells with poly(I:C) also stimulated a PKR-dependent increase in MIF production from MCF-7 cells. The biological consequence of the siRNA-induced increase in MIF production from MCF-7 cells was a PKR-dependent increase in proliferation of breast cancer cells. Furthermore, in cDNAs prepared from a primary human breast cancer cohort, we demonstrated a significant correlation (Spearman rank correlation coefficient, r = 0.50, p < 0.0001, n = 63) between PKR- and MIF-mRNA expression. In conclusion, this study highlights the potential biological consequences of off-target, nonspecific effects of siRNAs and underlines the safety concerns regarding the use of siRNAs in the treatment of human diseases, such as cancer. The Journal of Immunology, 2008, 180: 7125–7133.

Small interfering RNAs (siRNAs) represent a novel tool to induce gene silencing but are associated with a number of off-target, nonspecific, immunostimulatory effects (1–6). Because the discovery in 2001 that these duplexes of 21-nucleotide RNAs can mediate RNA interference in mammalian cells (7), several phase I clinical trials have already been completed to assess the efficacy of siRNAs in the treatment of human diseases such as age-related macular degeneration and respiratory syncytial virus infection (8). However, concerns regarding the use of siRNAs in vivo and the potential of off-target effects causing harm to individual patients remain outstanding.

Recognition of RNA by the immune system is dependent on RNA-sensing receptors including double-stranded RNA-dependent protein kinase (PKR), TLR3, TLR7, and TLR8, retinoic acid-inducible gene (RIG)-1 and melanoma differentiation-associated gene (MDA)5 (reviewed in Ref. 9). siRNAs are differentially recognized in immune- vs nonimmune cells. In immune cells such as plasmacytoid dendritic cells (pDCs), siRNAs delivered to the cell via cationic lipids or polycationic complexes are recognized in the endosome by TLR7 localized in the endosomal membrane (10). In contrast, in nonimmune cells, such as fibroblasts, siRNAs are recognized by PKR, RIG-I, and MDA5 in the cytoplasm, or TLR3 at the cell surface or in the endosome (9).

To date, reported off-target effects of siRNAs include sequence-independent activation of the type I IFN pathway (1, 3–5) in nonimmune cells. Transfection of cells with siRNAs has been shown to lead to IFN-mediated activation of the JAK/STAT pathway and global up-regulation of IFN stimulated genes. This effect is shown to be mediated in part by PKR (1, 3), a protein whose kinase activity can be regulated by dsRNA, polyamionic agents such as heparin (11), or PKR-activating protein (PACT) in the absence of dsRNA (12). The transcription factor, IFN-regulatory factor-3, is also activated in response to siRNA transfection and leads to the activation of genes independent of an IFN response (3). Other authors have reported enhanced TNF-α, IL-8, and HLA-DR expression (4) in addition to increased IFNα and IFNβ following siRNA treatment. In immune cells such as PBMCs, siRNAs exert immunostimulatory effects such as
increased TNF-α, IFN-γ and IL-6 via sequence-dependent mechanisms (6). Furthermore, non-specific repression of nontargeted mammalian genes has also been reported using siRNAs (2, 4). siRNAs, therefore, have the potential to cause diverse and non-specific effects when introduced into cells, in addition to silencing specific target genes.

siRNA technology provides an indispensable tool for functional genomic studies and is poised to play a major role in the development of therapeutics for human diseases (13, 14). In this context, macrophage migration inhibitory factor (MIF) represents a valuable therapeutic target for cancer using siRNA (reviewed in Ref. 15). MIF is a pleiotropic cytokine with well-described roles in cell proliferation (16), tumorgenesis, and angiogenesis (17). MIF has been shown to exert its actions via CD74 receptor binding (18) and activation of CD44 (19). Inhibition of MIF or MIF-binding receptor CD74 attenuates prostate cancer invasion (20), while enhanced CD44 activation has been shown to promote breast cancer cell invasion (21). CD44 has also been shown to be associated with adverse clinical prognosis not only in breast cancer but also in lung cancer, prostate cancer, and medulloblastoma (22). Furthermore, a role for MIF in tumorgenesis was demonstrated based on studies showing that the administration of MIF mAb in an animal model of B cell lymphoma resulted in markedly inhibited tumor growth and neangiogenesis (23). MIF also has the unique ability to stimulate sustained ERK1 and ERK2 MAPK activation in non-transformed cells leading to enhanced cell proliferation (16), while concurrently antagonizing the growth arrest and proapoptotic actions of the tumor suppressor, p53 (24, 25).

These novel functions for MIF place it in a unique position to promote the malignant progression of tumors. Therefore, in this study, we devised a siRNA strategy to target MIF in breast cancer, with the intention of attenuating cell proliferation in these cells. However, contrary to expectation, siMIF significantly enhanced expression of our target MIF gene at the RNA and protein level via a PKR-dependent mechanism. The biological consequence of this off-target effect was enhanced proliferation, rather than enhanced apoptosis, of breast cancer cells. This effect was also shown to be PKR dependent. This study highlights the importance of the non-specific, off-target effects of siRNAs having opposing biological effects than predicted and calls for more detailed analysis of such side-effects before the widespread introduction of larger scale human clinical trials using siRNA.

**Materials and Methods**

**Cell culture**

MCF-7, human breast adenocarcinoma cells (HTB-22; LGC Promochem) were cultured in MEM supplemented with 10% FBS (Invitrogen), 2 mM L-glutamine (Invitrogen), and antibiotics (complete medium). The pMIF-GFP vector was used to generate a stable cell line expressing MIF-GFP. pMIF-GFP consists of region 1–443 of the human MIF gene (GenBank: BC022414) shuttled into the EcoRI/BamHI sites of the pEGFPN2 vector (Clontech). In brief, to generate the stable MIF-GFP cell line, MCF7 cells were transfected with 700 ng of pMIF-GFP using FuGENE6 (Roche) in 6-well plates as per the manufacturer’s instructions. Transfected cells were incubated for 48 h in complete medium and then switched to a selective medium containing 1 mg/ml Geneticin (G418; Invitrogen) for 7 days and then up to 2 mg/ml G418 for another 3 wk. The MIF-GFP stably expressing cells leading to enhanced cell proliferation (16), while concurrently antagonizing the growth arrest and proapoptotic actions of the tumor suppressor, p53 (24, 25).

**siRNAs and transfections**

Human MIF siRNA was obtained from Dr. R. Mitchell (University of Louisville, Louisville, KY). Fluorescein control siRNA (siGFP) and human Lamin A/C siRNA (siLamin) were purchased from Qiagen. The non-targeting siRNA control (siControl) was obtained from Dharmacon Research. siGFP was used as the control siRNA in experiments where stated to monitor efficiency of gene silencing in MCF-7 cells expressing MIF-GFP.

**Analysis of MIF protein levels by ELISA**

Concentrations of MIF were determined in cell supernatants by ELISA using commercially available Ab pairs, specific for human MIF (Duoset; R&D Systems) according to the manufacturer’s instructions. In brief, 96-well Nunc Maxisorp microtiter plates (Invitrogen) were incubated overnight at 4°C with mouse anti-human MIF (2.0 μg/ml) in 50 μl PBS. After washing and blocking, a 50-μl sample or standards were added in triplicate to plates and incubated overnight at 4°C. After washing, 50 μl biotinylated goat anti-human MIF (200 ng/ml) was added to the plates and incubated for 2 h at room temperature. After washing, 50 μl streptavidin conjugated to horseradish-peroxidase (diluted 1/200) was added and incubated for 20 min at room temperature. The plates were washed and 50 μl o-phenylenediamine dihydrochloride (0.4 mg/ml; SIGMAFAST o-phenylenediamine dihydrochloride tablets; Sigma-Aldrich) in 0.05 M phosphate-citrate buffer containing H2O2 (0.4 mg/ml) was added to the wells. The reaction was stopped with 2N H2SO4 and the absorbance was measured at 492 nm. Recombinant MIF of known concentration and potency was used in the generation of standard curves. MIF values were calculated with reference to the standard curve and expressed as pg/ml. The sensitivity of the assay was 15 pg/ml. Ab pairs specific for human MIF were used in this assay and do not cross-react with other human proteins.

**Analysis of gene expression using quantitative real-time PCR (QPCR)**

For QPCR analysis, total RNA was extracted from MCF-7 cells using TRI Reagent (Sigma-Aldrich) according to the manufacturer’s instructions. First-strand cDNA (20 μl) was synthesized from 2 μg total RNA using oligo(dT) primers as described previously (29). An aliquot (2 μl) of each cDNA was used as template for PCR amplification with primers specific for MIF (sense primer: 5'-CCGGACAGGTCTACATCACTATTAC-3' and anti-sense primer: 5'-TGGGGCAGGTTGGAGGTCC-3'), PKR (sense primer: 5'-TCCAAATGACGGAGTCTTCCT-3' and anti-sense primer: 5'-CAGCACTATTCTTCTCCGGATCT-3') and the housekeeping gene, 18S ribosomal RNA (sense primer: 5'-GCCATGACGGAGTCTTCCT-3' and anti-sense primer: 5'-TGGGGCAGGTTGGAGGTCC-3'). PKR and the housekeeping gene, 18S ribosomal RNA (sense primer: 5'-GCCATGACGGAGTCTTCCT-3' and anti-sense primer: 5'-TGGGGCAGGTTGGAGGTCC-3'). PKR and the housekeeping gene, 18S ribosomal RNA (sense primer: 5'-GCCATGACGGAGTCTTCCT-3' and anti-sense primer: 5'-TGGGGCAGGTTGGAGGTCC-3'). PKR and the housekeeping gene, 18S ribosomal RNA (sense primer: 5'-GCCATGACGGAGTCTTCCT-3' and anti-sense primer: 5'-TGGGGCAGGTTGGAGGTCC-3'). PKR and the housekeeping gene, 18S ribosomal RNA (sense primer: 5'-GCCATGACGGAGTCTTCCT-3' and anti-sense primer: 5'-TGGGGCAGGTTGGAGGTCC-3'). PKR and the housekeeping gene, 18S ribosomal RNA (sense primer: 5'-GCCATGACGGAGTCTTCCT-3' and anti-sense primer: 5'-TGGGGCAGGTTGGAGGTCC-3'). PKR and the housekeeping gene, 18S ribosomal RNA (sense primer: 5'-GCCATGACGGAGTCTTCCT-3' and anti-sense primer: 5'-TGGGGCAGGTTGGAGGTCC-3'). PKR and the housekeeping gene, 18S ribosomal RNA (sense primer: 5'-GCCATGACGGAGTCTTCCT-3' and anti-sense primer: 5'-TGGGGCAGGTTGGAGGTCC-3'). PKR and the housekeeping gene, 18S ribosomal RNA (sense primer: 5'-GCCATGACGGAGTCTTCCT-3' and anti-sense primer: 5'-TGGGGCAGGTTGGAGGTCC-3'). PKR and the housekeeping gene, 18S ribosomal RNA (sense primer: 5'-GCCATGACGGAGTCTTCCT-3' and anti-sense primer: 5'-TGGGGCAGGTTGGAGGTCC-3'). PKR and the housekeeping gene, 18S ribosomal RNA (sense primer: 5'-GCCATGACGGAGTCTTCCT-3' and anti-sense primer: 5'-TGGGGCAGGTTGGAGGTCC-3'). PKR and the housekeeping gene, 18S ribosomal RNA (sense prime
Fugene 6 (Roche, Basel, Switzerland) according to the manufacturer’s instructions; each well received 1.0 μg of either pMIF-1021-Luc (30), pLuc-IFN-β (31), or pSRE-Luc (Stratagene) inducible reporter constructs and 1.0 μg of pTK-Rluc control vector for normalization of transfection efficiency. After 4 h, cells were split into split into six wells of a 24-well plate. Cells were then cultured overnight before transfecting with RNAiFect alone or siMIF complexed to siMIF. Cells were then cultured for a further 24 h before lysing and luciferase activity was determined in relation to renilla activity using a Turner Design 20/20 luminometer (Turner Biosystems). Values obtained were expressed in relative light units.

**Proliferation assay**

MCF-7 cells were transfected with either RNAiFect alone or siRNAs complexed with RNAiFect, as described above, and incubated for 48 h. On completion of the experiment, cell culture supernatants were removed from each monolayer and transferred, aseptically, to a corresponding well of a 24-well plate containing naive MCF-7 cells at 80% confluency. Cells were then cultured for a further 24 h before the addition of 0.5 μCi/well [3H]thymidine (Amersham Biosciences). Following an additional 24 h of culture, cells were then harvested and proliferation was assessed by [3H]thymidine incorporation. In brief, cell supernatants were removed and monolayers washed with PBS before incubating with 10% trichloroacetic acid for 1 h at 4°C. Trichloroacetic acid was then removed and cells were washed before incubation with 0.1M NaOH for 30 min at room temperature on a shaker. Cells lysates were then prepared and transferred into Ecoscint A scintillation solution (National Diagnostics, Hull, U.K.), vortexed, and counted on a liquid scintillation analyzer (Packard). Proliferation was recorded per sample as dpm/well.

**Primary human breast cancer cohort**

RNA from 63 primary human breast cancer samples (32) was donated by Dr. A. McCann and Dr. W. Y. Chung (The Conway Institute for Molecular and Biomedical Research, Dublin, Ireland). RNA obtained was then reversed transcribed using random priming and SuperScript II reverse transcriptase, as described above. Complementary DNA generated was then used as a template for quantitative real-time PCR with primers specific for PKR, MIF, or 18S, as described above.

**Statistical analysis**

Student’s *t* test was used to test for statistical differences between two experimental groups. Mann-Whitney *U* test was used to test for statistical significance between two groups where sample populations were not normally distributed. One-way ANOVA was used to test for statistical significance between experimental groups of three. Multiple comparisons between groups were then assessed using the Tukey-Kramer post hoc test. Correlation of PKR mRNA with MIF mRNA expression was assessed using the nonparametric Spearman rank correlation assay (correlation coefficient: *r*). Statistical significance for all tests was recorded at *p* < 0.05.

**Results**

siMIF and control siRNAs increase MIF production from mammary adenocarcinoma (MCF-7) cells expressing MIF-GFP in a dose-dependent manner

siRNA-based therapeutic strategies present a novel tool to treat human diseases, including breast cancer. MIF provides a candidate gene to target with such a technology for treatment of breast cancer because of its known roles in cell proliferation, tumorigenesis, and angiogenesis. We therefore designed an anti-MIF siRNA strategy to knockdown MIF expression in MCF-7 cells expressing MIF-GFP. Silencing of the MIF gene in MCF-7 MIF-GFP cells was observed between 48 and 96 h posttransfection (data not shown). However, an investigation of MIF protein levels in supernatants from siRNA-transfected cells revealed increased MIF production from these cells, compared with mock-transfected cells. This effect was shown to be dose-dependent over a concentration range of 25–50 nM for siMIF (Fig. 1A). A significant increase in MIF production was observed at 48 h posttransfection with 25 nM siMIF (*p* < 0.001) and 50 nM siMIF (*p* < 0.001) compared with mock controls (Fig. 1A). In addition, transfection of the control siRNAs, siGFP, and siLamin A/C into MCF-7 cells significantly increased MIF production from cells compared with mock controls (siGFP, siMIF, and siLamin A/C vs mock-transfection, *p* < 0.001; siLamin A/C-transfection vs mock-transfection, *p* < 0.01; Fig. 1B). These results suggest that the siRNA-induced increase in MIF production is sequence independent as it is evident when using a number of different siRNAs of unrelated sequences. To investigate whether these effects are unique to the MCF-7 cell line, we investigated the production of MIF from an alternative cell line and using additional siRNA sequences. We demonstrated an increase in MIF production from a primary human lung fibroblast cell line (CCD-19Lu cells) following transfection with 50 nM early growth response-1 siRNA (312.8 ± 26.2 pg/ml; mean ± SEM; MIF) and 50 nM siControl (384 ± 4 pg/ml; mean ± SEM; MIF; *p* < 0.05) compared with mock-transfected cells (259.5 ± 9.4 pg/ml; mean ± SEM; MIF). These results suggest that increased MIF production following siRNA transfection is not restricted to the MCF-7 cell line.

**Transfection of MCF-7 cells with siMIF increases MIF and PKR transcription**

We observed knockdown of the MIF gene using siMIF in MCF-7 cells between 48 and 96 h using Northern blot (data not shown). In this study, we demonstrate, at 24 h posttransfection, a significant increase (*p* < 0.05) in MIF mRNA expression following treatment of MCF-7 cells with siMIF using qPCR compared with mock controls (Fig. 2A). Furthermore, a significant increase (*p* < 0.01) in
MIF-luciferase activity was also observed at 24 h posttransfection with siMIF compared with mock-transfection (Fig. 2B). Other authors have demonstrated that the off-target, nonspecific effects of siRNA are PKR-dependent (1, 3). In this study, we investigated the effect of siMIF treatment on PKR mRNA expression and demonstrated that siMIF-transfection significantly enhances PKR mRNA expression in MCF-7 cells compared with mock-transfection at 24 h (p < 0.05) using qPCR (Fig. 2C). In addition, we investigated the effect of siRNA-transfection on MIF transcription in an alternative cell line and using additional siRNA sequences to assess the cell-specificity of our siRNA effect. We demonstrated an increase in MIF transcription using qPCR in CCD-19Lu cells following transfection with 50 nM early growth response-1 siRNA (1.57 ± 0.13; mean ± SEM; MIF mRNA per unit 18S mRNA; p < 0.05) compared with control cells (0.99 ± 0.9; mean ± SEM; MIF mRNA per unit 18S mRNA). These results suggest that increased MIF transcription following siRNA transfection is not restricted to the MCF-7 cell line or the siMIF sequence used in this study and may have implications for other cell types.

**Increased MIF production following siRNA transfection is PKR dependent**

Having demonstrated that siMIF transfection increases PKR transcription in MCF-7 cells, we then investigated the role of PKR in siRNA-induced MIF secretion in these cells. Cells were pretreated for 45 min with 2 mM 2-AP, before transfection with either siMIF or siControl for 48 h. siMIF-induced MIF secretion was found to be significantly attenuated in the presence of 2-AP (p < 0.05) compared with siMIF transfection alone (Fig. 3). This result suggests a role for PKR in MIF production in MCF-7 cells following siRNA transfection.

**MIF production from MCF-7 cells transfected with poly(I:C) is stimulated in a dose-, time-, and PKR-dependent manner**

siRNAs in nonimmune cells are recognized by PKR, RIG-I, MDA5, and TLR3. Cytoplasmic dsRNA is sensed by PKR, RIG-I, and MDA5 (33). We therefore investigated whether treatment of MCF-7 cells with the synthetic dsRNA analog, poly(I:C), could mimic siRNA-induced MIF production from MCF-7 cells. When poly(I:C) was added in culture to MCF-7 cells (1–100 μg/ml), no significant increase in MIF production from MCF-7 cells was observed compared with MCF-7 cells in medium alone (Fig. 4A; closed bars). However, when poly(I:C) was complexed with RNAiFect and transfected into cells, as described previously (26, 27, 28), a significant increase in MIF production from MCF-7 cells was observed compared with mock-transfected cells. This effect was dose-dependent over a concentration range of 1–100 μg/ml poly(I:C) (1–100 μg/ml, p < 0.001; Fig. 4A; open bars). In addition, MIF production from MCF-7 cells following transfection with poly(I:C) was significantly increased compared with cells

**FIGURE 2.** Transfection of MCF-7 cells with siMIF increases MIF mRNA expression (A), pMIF-1021 luciferase activity (B), and PKR mRNA expression (C). Cells were transfected with RNAiFect alone or 50 nM siMIF complexed with RNAiFect, and then cultured for 24 h. A, MIF mRNA and PKR mRNA expression (C) were determined by QPCR. B, pMIF-1021 luciferase promoter activity was assessed using a dual luciferase assay kit. A and C, Values obtained for QPCR represent mean ± SEM for MIF or PKR mRNA and are expressed per unit of 18S ribosomal mRNA. B, Values obtained for luciferase assays represent mean ± SEM and are expressed in relative light units (RLU). *, p < 0.05; siMIF-transfection vs mock-transfection. **, p < 0.01; siMIF-transfection vs mock-transfection.

**FIGURE 3.** siRNA-induced MIF production from MCF-7 cells is attenuated in the presence of a PKR inhibitor. MCF-7 cells were pretreated with 2 mM 2-AP for 45 min before transfection with RNAiFect alone, 50 nM siMIF, or 50 nM siControl, complexed with RNAiFect, and cultured for 48 h. MIF protein levels in cell supernatants were quantitated by ELISA. Values represent mean ± SEM of MIF protein expressed in pg/ml. *, p < 0.05; siMIF transfection vs siMIF transfection with pretreatment with a PKR inhibitor.
treated with poly(I:C) alone (1–100 μg/ml, p < 0.001; Fig. 4A). Previous authors have demonstrated that introduction of Poly(I:C) directly into the cytoplasm of embryonic fibroblasts via transfection led to the induction of IFN-α and IFN-β in a TLR3-independent manner (26). The increase in MIF production from MCF-7 following poly(I:C) transfection was shown to be significantly increased in a temporal manner over 24–48 h compared with mock controls (p < 0.001; Fig. 4B). In addition, when MCF-7 cells were pretreated with the PKR inhibitor, 2-AP, a significance decrease in poly(I:C)-driven MIF production was observed compared with mock-transfected controls (p < 0.001; Fig. 4C).

**FIGURE 4.** MIF production is stimulated in MCF-7 cells transfected with poly(I:C) in a dose- (A) and time-dependent (B) manner, and is attenuated in the presence of a PKR inhibitor (C). A, MCF-7 cells were treated with 1–100 μg/ml poly(I:C) alone (- RNAiFect; closed bars) or poly(I:C) in complex with transfection reagent (+ RNAiFect; open bars). B, MCF-7 cells were transfected with RNAiFect alone (mock) or poly(I:C) (0.1 μg/ml) and cells were cultured for 24–48 h thereafter. C, MCF-7 cells were pretreated with 2 mM 2-AP for 45 min before transfection with RNAiFect alone or poly(I:C) (0.1 μg/ml), cells were then cultured for 48 h. MIF protein levels in cell supernatants were quantitated by ELISA. Values represent mean ± SEM of MIF protein expressed in pg/ml. XXX, p < 0.001; cells transfected with poly(I:C) vs mock-transfected cells. ++, p < 0.001; cells treated with poly(I:C) complexed with RNAiFect vs cells treated with poly(I:C) alone. +++, p < 0.001; cells transfected with poly(I:C) vs cells pretreated with a PKR inhibitor and then transfected with poly(I:C).

**FIGURE 5.** Poly(I:C) but not siRNAs stimulate IFN-β- and ISRE-dependent transcription in MCF-7 cells. C, Treatment of MCF-7 cells with recombinant IFN-β does not stimulate MIF production. MCF-7 cells expressing IFN-β (A) or ISRE luciferase reporter (B) constructs were co-transfected with RNAiFect alone (mock), poly(I:C) (0.1 μg/ml), siMIF (50 nM), or siGFP (50 nM). Luciferase reporter gene activity was measured after 24 h using the luciferase dual assay kit. C, MCF-7 cells were treated with 10–1000 IU/ml recombinant IFN-β for 24–48 h. MIF protein levels in cell supernatants were then quantitated by ELISA. A and B, Values obtained for luciferase assays represent mean ± SEM and are expressed as fold-increase in promoter activity compared with mock-transfected cells. C, Values obtained for ELISAs represent mean ± SEM of MIF protein expressed in pg/ml.
siRNA-induced MIF production from MCF-7 cells is not stimulated by IFN-β

To date, reported off-target effects of siRNAs in nonimmune cells include sequence-independent activation of the type I IFN pathway (1, 3–5). This effect is shown to be mediated in part by PKR (1, 3). In this study, we investigate whether the siRNA-induced MIF production in MCF-7 cells is IFN-dependent. Activation of IFN-β and IFN-stimulated response element (ISRE)-dependent transcription was assessed in MCF-7 cells in response to siRNA transfection. Cells transfected with poly(I:C) served as a positive control. Poly(I:C), but not siMIF or siGFP, activated both IFN-β (Fig. 5A) and ISRE-dependent (Fig. 5B) transcription in luciferase reporter assays. Furthermore, addition of recombinant IFN-β (10–1000 IU/ml) to MCF-7 cells for 24–48 h did not stimulate MIF production from MCF-7 cells (Fig. 5C). These results suggest that siRNA-induced MIF production is not IFN-β-dependent.

siRNA treatment is associated with enhanced cell proliferation and is PKR mediated

MIF plays a pivotal role in cell proliferation. In this study, we investigated the effect of siRNA-induced MIF on proliferation of naïve recipient cells. Transfer of supernatants from siMIF- or siControl-treated cells to naïve cells led to a significant increase in cell proliferation in recipient cells (p < 0.01, siMIF v mock; p < 0.01, siControl v mock) compared with transfer of supernatants from mock-transfected cells (Fig. 6). Furthermore, studies of human malignancies and tumor cell lines suggest that, in general, patients bearing tumors with a higher PKR content have a more favorable prognosis (34). However, in human breast carcinoma cells, dysregulation of PKR may be associated with the establishment or maintenance of the transformed state (34). Therefore, we investigated the role of PKR in mediating the siRNA-induced increase in proliferation. The presence of a PKR-inhibitor significantly attenuated increases in proliferation in naïve cells following transfer of supernatants from siMIF-treated cells (p < 0.01) or siControl-treated cells (p < 0.001) vs mock controls (Fig. 6). These results imply that there is a PKR-dependent elaboration of proproliferative cell products from siRNA-transfected cells that are active on naïve breast cancer cells.

A significant correlation exists between PKR and MIF primary human breast cancer

We have demonstrated a role for PKR in the regulation of MIF in the MCF-7 breast carcinoma cell line. In this study, we investigate the correlation between PKR and MIF mRNA expression in a cohort of 63 primary human breast cancer cDNA samples. We observed a significant correlation between PKR and MIF mRNA expression in these patients (Fig. 7; Spearman correlation coefficient, r = 0.5035; p < 0.0001). This finding supports our hypothesis that MIF is regulated by PKR.

Discussion

The major findings of this study are previously unreported off-target, nonspecific effects of siRNA technology, namely enhanced MIF production from human breast cancer cells and an increase in proliferation of these cells. Previously reported off-target effects of siRNAs include activation of type I IFNs and the JAK/STAT pathway, resulting in stimulation of IFN-responsive genes (1, 3–5). Other authors have reported additional effects, including activation of proinflammatory cytokines (4, 6) and IRF-3-mediated effects, which are IFN-independent (3). In this study, siRNA-induced MIF production was not found to be IFN dependent. siRNAs and dsRNA are recognized in the cytoplasm via PKR, RIG-I, and MDA-5 (33). In this study, we demonstrated in addition that transfection of MCF-7 cells with poly(I:C) induced MIF production from these cells. MCF-7 breast carcinoma cells express unusually high levels of PKR compared with normal human breast cell lines, including MCF10A (35). Therefore, we investigated the role of PKR in mediating siRNA- and poly(I:C)-induced MIF production in these cells. In this study, we report that increased MIF production from MCF-7 cells in response to siRNA- or poly(I:C)-transfection are...
PKR-dependent. In addition, the siRNA-induced increase in cell proliferation in MCF-7 cells was also mediated by PKR. These data are in agreement with previous studies, which state that off-target, non-specific effects of siRNAs are partly PKR mediated (1, 3) and highlight the broad and complicating effects of introducing siRNAs into cells. In addition, these results have implications for the use of siRNA-based therapeutic strategies in vivo for breast cancer.

Numerous studies document the off-target and nonspecific effects of siRNA in vitro. Both nonspecific stimulation (1–5) and repression (2, 4) of nontarget genes has been reported and activation of the type I IFNs, the JAK/STAT signaling pathway, and IFN-responsive genes are the most widely reported effects from in vitro studies (1, 3–5). Fewer studies have addressed the consequences of siRNA delivery in vivo. In 2003, Sioud and colleagues (36) reported activation of the immune system and cytokine production following cationic-liposome delivery of siRNA to adult mice. In other studies, mice that were injected with immunostimulatory siRNAs displayed signs of toxicity including, elevated levels of serum alanine and aspartate aminotransferases, along with reduced numbers of lymphocytes and platelets (6, 37). siRNAs, therefore, have been demonstrated to induce unwanted and potentially deleterious effects when they are used either in vitro or in vivo, in addition to silencing target genes. However, siRNA represents a novel technique to silence genes that are critically involved in the pathogenesis of human disease.

In this study, we considered MIF as a candidate therapeutic target for breast cancer using siRNA technology, as MIF has been well described to promote a malignant phenotype by its ability to increase cell proliferation, promote tumorogenesis, and stimulate angiogenesis (reviewed in Ref. 15). For this purpose, we devised a siRNA strategy targeting the MIF gene. In addition to gene silencing, we observed a dose-dependent increase in secretion of MIF from MCF-7 cells. These findings are previously unreported but are in agreement with earlier studies that have reported that the off-target effects of siRNAs are concentration dependent (2). In addition, we also demonstrated that these effects are nonspecific as various controls siRNAs, including siGFP, siControl, and siLamin A/C, also stimulated the production of MIF from MCF-7 cells. This finding is in agreement with other authors who demonstrate that the off-target effects in nonimmune cells are both nonspecific and sequence independent (4). In contrast, in immune cells such as pDCs, siRNAs delivered to the cell via cationic lipids or polycationic complexes are recognized in the endosome in a sequence-dependent manner by TLR7 (10). In addition, Judge et al. (6) have recently demonstrated that siRNAs containing GUGU or poly(U) motifs are more immunostimulatory in PBMCs and therefore, that the off-target effects of siRNAs in these cells are sequence dependent. In this study, we demonstrated robust silencing of the MIF gene in MCF-7 cells expressing MIF-GFP at 48 h posttransfection and thereafter (data not shown). However, at the earlier time-point of 24 h post-siRNA-transfection, a significant increase in MIF transcription was observed using both QPCR and reporter assays. We therefore propose that the increase in MIF protein following siRNA-transfection is partly dependent on de novo synthesis of MIF.

Recent studies have demonstrated that RNA is recognized by the immune system by sensors including PKR, TLR3, TLR7, and TLR8, RIG-I and MDA5 (reviewed in Ref. 9). Specifically, siRNAs are recognized in immune cells such as pDCs by endosomal TLR7 and mediate sequence-dependent effects. In contrast, in nonimmune cells, siRNAs induce nonspecific, sequence-independent effects. Candidate RNA sensing receptors for siRNAs in nonimmune cells include PKR, RIG-I, MDA5, and TLR3.

Early studies investigating the off-target, nonspecific effects of siRNAs determined a role for PKR in mediating these side-effects (1, 3). Specifically, PKR has previously been shown to mediate the type I IFN-dependent effects of siRNAs. A range of biological processes is affected by PKR including cell-growth, -differentiation, and -apoptosis, in addition to virus multiplication. PKR is present at low constitutive levels in cells and its expression is classically induced by IFN. Activation of the kinase activity of PKR requires binding to an activator protein. The most well-characterized activator is dsRNA, although other polyanionic agents such as heparin have also been shown to activate PKR in vitro (11). PACT can also activate PKR kinase activity in the absence of a source of dsRNA, thereby playing an important role in PKR activation in response to cellular stress (12). Upon activation, PKR is autophosphorylated and phosphorylates its substrates, which include the α-subunit of the eukaryotic protein synthesis initiation factor, eIF2α. Phosphorylated eIF2α entraps another initiation factor, eIF2B, in an inactive complex, which subsequently causes inhibition of translation. PKR also phosphorylates other proteins including IκB, p53, NF90, B56α, HIV-1 TAT, RNA helicase A, and histone (reviewed in Ref. 38). PKR promotes phosphorylation of IκB via the IKK complex and thus activates transcription factor NF-κB (39).

In this study, we demonstrated firstly, that siMIF increased PKR transcription in MCF-7 cells following introduction to cells. We then demonstrated that siRNA-induced MIF production from MCF-7 cells was PKR-dependent using the PKR inhibitor, 2-aminopurine. This inhibitor has been used in several studies to abrogate the effects of PKR (4, 40–42). In support of this finding, we also demonstrated an increase in MIF production from MCF-7 cells, following transfection with poly(I:C), which could be attenuated in the presence of the 2-AP, PKR inhibitor. Other authors suggest that introduction of poly(I:C) into the cytoplasm via transfection leads to TLR-3-independent activation of type I IFNs (26). This may suggest that poly(I:C)-induced MIF is induced in MCF-7 cells by a TLR3-independent mechanism or via an IFN-dependent mechanism. We did not observe a difference in MIF production from TLR3 expressing human embryonic kidney-293T (HEK293T) cells compared with HEK293T cells following siRNA transfection (data not shown), suggesting that poly(I:C)-induced MIF may not be TLR3-dependent. In addition, poly(I:C), but not siRNA, stimulated IFN-β and ISRE-dependent transcription in MCF-7 cells in this study, but treatment of MCF-7 cells with recombinant IFN-β did not stimulate MIF production from these cells. Therefore, we suggest the siRNA- or poly(I:C)-induced MIF production from MCF-7 cells is IFN-independent but occurs via a PKR-dependent and TLR3-independent mechanism.

A recent study by Das et al. (43) demonstrated that members of the Sp protein family of transcription factors regulate PKR transcription, in the absence of a source of IFN. Specifically, activation of human PKR mRNA transcription was shown to be regulated, in the absence of IFN, by the cooperative actions of Sp1 and Sp3 proteins on a kinase conserved sequence DNA element located within the PKR promoter (43). We suggest that Sp proteins may play a role in mediating the siRNA-induced, and IFN-independent, increase in PKR mRNA expression observed in this study.

PKR must bind one of its coactivators to become biologically active. In this study, we propose that siRNA may be activating PKR by either direct- or indirect-mechanisms. Several studies have demonstrated that siRNA can directly bind to and activate PKR (1, 44, 45). Recently, Pathnveetil et al. (45) demonstrated that PKR’s kinase activity is stimulated in vitro 3- to 5-fold by 19 bp siRNAs with 2nt 3’-overhangs, whereas poly(I:C) induced a 17-fold increase in kinase activity in the same conditions. However, studies by Marques et al. have demonstrated that 21–27 bp siRNAs activated PKR in vitro but were poor activators in vivo following...
transfection into cultured cells (44). Furthermore, in vitro synthesized siRNAs, but not synthetic siRNAs, have been shown to activate PKR directly (46). Therefore, evidence for direct activation of PKR by siRNAs is conflicting. Alternatively, the cellular protein, PACT, may indirectly regulate PKR activity by siRNAs. PACT has been shown to activate PKR in the absence of dsRNA and its expression is not regulated by IFN or dsRNA. However, PACT can also bind dsRNA to activate PKR. Therefore, PACT can regulate PKR in the presence or absence of dsRNA, with PACT-PKR binding leading to activation of PKR by autophosphorylation (47). Recently, PACT was shown to be essential in siRNA-induced RNA interference (48). In their study, PACT was identified as another dsRNA-binding protein that functions as a component of the human RNA-induced silencing complex (RISC) (48). These authors propose that the component(s) of RISC may be regulated by PKR through phosphorylation and that PACT and HIV TAR RNA-binding protein (an inhibitor of PKR) may regulate PKR activity to control RISC activity (48). Therefore, we suggest in this study that entry of siRNAs into the RISC complex may alter the regulation of PKR by PACT (and HIV TAR RNA-binding protein), thus resulting in PKR autophosphorylation.

PKR has been shown to regulate activity of NF-κB via its interaction with the IκB complex and can therefore regulate genes that are NF-κB-dependent (39). In this study, we propose that PKR-induced activation of NF-κB may act to enhance MIF transcription in response to siRNA transfection of MCF-7 cells. In support of this hypothesis, using gene promoter analysis, we have demonstrated the presence of a NF-κB binding site at position −513 of the human MIF promoter. In addition, we have previously reported that overexpression of the adaptor, TIR-domain-containing adapter-inducing IFN-β-related adapter molecule in HEK cells resulted in an increase in NF-κB and IFN-β-luciferase activity (49) and subsequently, MIF production from these cells (data not shown). In addition, using chromatin immunoprecipitation assay analysis, we have also demonstrated that NF-κB binds to the MIF promoter (data not shown). Furthermore, other authors have demonstrated that TNF-α can induce MIF production in macrophages via NF-κB and JNK and that this leads to increased invasive capacity of tumor cells in tumor-macrophage cocultures (50). These findings support our hypothesis that MIF mRNA expression is increased following siRNA transfection in a PKR-NF-κB-dependent manner and that this effect is IFN independent. We have also shown that treatment of MCF-7 cells with recombinant IFN-β does not stimulate MIF production from these cells and this confers with our hypothesis.

PKR plays an important role in mediating the antiviral effects of IFNs, however, in uninfected cells PKR is also implicated in regulating cell proliferation under normal conditions (38). PKR plays a specific role in breast carcinoma cells, this kinase is usually associated with cell death but in breast cancer it has been shown to promote and enhance the malignant phenotype (35, 51, 52). Haines et al. (53) demonstrated in 1996 that human invasive ductal breast carcinomas exhibit high levels of PKR. Levels of PKR are also unusually high in MCF-7 cells compared with normal human breast cell lines (35). This may be due to the fact that MCF-7 cells have higher PKR mRNA levels and exhibit increased transcription from the PKR promoter compared with nontransformed cell lines such as MCF10A (52). In addition, breast carcinoma cells lines have higher endogenous PKR kinase activity and higher levels of phosphorylated eIF2α than nontransformed epithelial cells line (51). This is partly due to the fact that nontransformed cell lines contain p58, an inhibitor of PKR that interacts with PKR in these cells and may be responsible for their low PKR activity. Due to the known role of MIF in cell proliferation and the role of PKR human breast cancer, we questioned the functional significance of the siRNA-induced, and PKR-dependent, increase in MIF in MCF-7 cells. We investigated the effect of siRNA-induced MIF on proliferation of MCF-7 cells and investigated the role of PKR. Following the transfer of supernatants from MCF-7 cells transfected with siRNA to naive cells, we observed an increase in proliferation in recipient MCF-7 cells. Furthermore, pretreatment of MCF-7 cells with a PKR inhibitor before transfection with siRNA, resulted in a significant decrease in proliferation in recipient MCF-7 cells. Therefore, our data demonstrate a biological consequence for a siRNA-induced off-target effect, namely a PKR-associated increase in proliferation of human breast cancer cells. We hypothesize that siRNA induces the production of MIF from transfected cells and that MIF subsequently acts on adjacent cells to enhance proliferation via a p44/p42 ERK mitogen-activated protein MAPK (p44/p42 ERK MAP kinase)-dependent mechanism. Other authors have demonstrated that both endogenously secreted and exogenously added MIF stimulates the proliferation of NIH3T3 cells, and this response is associated with the activation of p44/p42 ERK MAPK (16). In support of our hypothesis, we have shown that addition of exogenous MIF to MCF-7 cells leads to sustained p44/p42 ERK MAPK activation over a 24 h period (data not shown).

We also investigated the correlation between PKR mRNA and MIF mRNA expression, a cohort of 63 primary human breast cancer cDNAs, and found a significant correlation between PKR and MIF-mRNA expression. This correlation also supports our hypothesis that MIF is regulated in a PKR-dependent manner. Other authors have reported that breast cancer cells express elevated levels of both PKR (53) and MIF (54). However, we provide evidence for the first time of a direct correlation between PKR and MIF in primary human breast cancer cells. We suggest that in these cells, PKR acts to enhance MIF expression via an NF-κB-dependent mechanism. In the absence of a source of dsRNA to activate PKR in vivo, we propose that PACT may be activating PKR in breast cancer cells and hence, increasing MIF transcription in these cells via an NF-κB-dependent mechanism. In support of this hypothesis and a role for PACT, recent studies have demonstrated that PACT is up-regulated in small-sized peripheral adenocarcinomas of the lung and that this is associated with shorter survival periods (55). In addition, higher levels of PACT were also reported in squamous cell carcinoma of the lung (56).

In conclusion, we identify the activation of PKR-dependent MIF in response to siRNA transfection as a previously unreported side effect, and associate this effect with enhanced cell proliferation. We provide evidence that both siRNA and synthetic dsRNA can stimulate MIF production in a PKR-dependent manner. We also demonstrate siRNA-induced MIF mRNA and protein in an additional cell line, namely, a primary human lung fibroblast cell line. This result implies that this off-target effect is not restricted to the MCF-7 cell line and may have implications for additional cell types. We also demonstrate a significant correlation between PKR and MIF in primary breast cancer samples and propose a role for PKR-dependent MIF in breast cancer. In conclusion, this study highlights the prevalence of unwanted and nonspecific effects of siRNAs. These data also emphasize the importance of more clearly defining the off-target effects of siRNAs and support a more cautionary approach to the use of siRNAs as a widespread therapeutic strategy, particularly in breast cancer.

Disclosures
The authors have no financial conflict of interest.