Forkhead box O1 (FOXO1) controls the migratory response of Toll-like receptor (TLR3)-stimulated human mesenchymal stromal cells

Sun Hwa Kim1†, Amitabh Das2‡, Hae In Choi3†, Ji Hoon Kim1, Jin Choul Chai1,4, Mi Ran Choi2‡, Bert Binas1, Kyung Sun Park2, Young Seek Lee1, Kyung Hwa Jung2, Young Gyu Chai1,3*

From the 1Department of Molecular & Life Science, Hanyang University, Ansan, 15588, Republic of Korea, the 2Institute of Natural Science & Technology, Hanyang University, Ansan, 15588, Republic of Korea, the 3Department of Bionanotechnology, Hanyang University, Seoul, 04673, Republic of Korea

†Equal contributors

Running title: FOXO1 controls hMSCs migration to TLR3 stimulation

ABSTRACT

Mesenchymal stromal cells (MSCs) can potently regulate the functions of immune cells and are being investigated for the management of inflammatory diseases. Toll-like receptor 3 (TLR3)-stimulated human MSCs (hMSCs) exhibit increased migration and chemotaxis within and toward damaged tissues. However, the regulatory mechanisms underlying these migratory activities are unclear. Therefore, we analyzed the migration capability and gene expression profiles of TLR3-stimulated hMSCs using RNA sequencing, wound healing, and transwell cell migration assay. Along with increased cell migration, the TLR3 stimulation also increased the expression of cytokines, chemokines, and cell migration–related genes. The promoter regions of the latter showed an enrichment of putative motifs for binding the transcription factors forkhead box O1 (FOXO1), FOXO3, nuclear factor kappa B (NF-κB1), and RELA proto-oncogene, NF-κB subunit. Of note, FOXO1 inhibition by FOXO1-selective inhibitor AS1842856 significantly reduced both migration and the expression of migration-related genes. In summary, our results indicate that TLR3 stimulation induces hMSC migration through the expression of FOXO1-activated genes.

INTRODUCTION

Mesenchymal stromal cells (MSCs) have the capacity to differentiate into osteocytes, adipocytes, and chondrocytes (1-3). MSCs are clinically available for use in the repair and regeneration of injured tissue and can be isolated from many tissues and expanded ex vivo (4-6). MSCs are able to modulate immune cells and
FOXO1 controls hMSC migration to TLR3 stimulation

immunosuppressive properties, which makes them a potential therapeutic. MSCs play a role as immune modulators by secreting soluble factors and regulating immune cells (7-10). These immunomodulatory properties can be used for the treatment of inflammatory diseases such as autoimmune-induced inflammatory bowel diseases and graft-versus-host disease (11). Several studies have suggested that the immunomodulatory properties of MSCs contribute to their beneficial therapeutic effects (12-16).

Toll-like receptors (TLRs) play a crucial role in the recognition of pathogens (17,18) and initiate downstream signaling leading to an inflammatory response (17-21). The TLR family recognizes several types of pathogens, such as the bacterial lipoprotein peptidoglycan (PGN), which is recognized by TLR2; viral double-stranded RNAs and their DNA analogs (poly(I:C)), which are recognized by TLR3; and lipopolysaccharides (LPSs) from Gram-negative bacteria, which are recognized by TLR4 (22-24). In MSCs, TLRs play an essential role in immune modulation (18,19). Several studies have suggested that the immunomodulatory effects of human bone marrow MSCs (hBM-MSCs) are regulated through the activation of TLRs. Specifically, the activation of TLR3 and TLR4 induces pro-inflammatory or anti-inflammatory responses and mediates immunosuppressive effects (2-4,25,26). In addition, activated TLRs modulate MSC proliferation, differentiation, and migration, but these effects differ according to the tissue and species from which the MSCs are derived (23).

One of the most important features in the therapeutic applications of MSCs is the homing of transplanted MSCs into inflammation sites within damaged tissues (4,27). Transplanted MSCs can migrate to injured sites and promote the repair process through their immunomodulatory activities (4,28). Migrated MSCs release pro-inflammatory or anti-inflammatory factors and regulate immune cells (16,29-33). Conversely, chemokines and cytokines of various origins, including stromal cell-derived factor-1α (SDF-1α) (34-36), hepatocyte growth factor (HGF) (37), and chemokine (C-C motif) ligand 2 (CCL2) (27,38), induce migration of MSCs. Also, activation of TLR3 stimulates the secretion of immune modulators and soluble factors that lead to immunosuppressive responses (2,25). Several studies have suggested that stimulation of TLR3 regulates migration properties and immunomodulatory factors, including indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), and transforming growth factor β (TGFβ) (2,26,39). However, the mechanism of the TLR3-activated migration of hMSCs is unknown. Therefore, we investigated whether TLR3-stimulated hMSCs contribute to the pathway in response to hMSC migration using gene expression profiling.

In this study, we performed RNA-sequencing for gene expression profiling of hMSCs treated with a TLR3 ligand (poly(I:C), polyinosinic:polycytidylic acid) in comparison with unstimulated hMSCs (control hMSCs). We analyzed differentially expressed genes and validated the RNA-seq data using quantitative real-time PCR (qRT-PCR). Our results show that TLR3-stimulated hMSCs express inflammatory- and migration-responses-related genes, thus revealing the molecular effects of TLR3 activation. Additionally, our results show that the TLR3-stimulated hMSCs increased cell migration through the activation of forkhead box protein O1 (FOXO1). Together, these results strengthen the molecular foundation for the clinical utilization of the cell migration abilities of hMSCs.

RESULTS

Characterization of TLR3-stimulated hMSCs

To study the effects of TLR3 stimulation on hMSCs, we incubated them with polyinosinic:polycytidylic acid (poly(I:C), TLR3 ligand) for 4 h. Non-stimulated hMSCs (control hMSCs) and TLR3-stimulated cells (TLR3-stimulated hMSCs) exhibited a similar spindle-shaped fibroblastic morphology (Fig. 1A). Flow cytometry expression analysis of the typical MSC-related surface antigens showed that both the control hMSCs and TLR3-stimulated hMSCs were positive for CD29, CD44, CD73 and CD105.
FOXO1 controls hMSCs migration to TLR3 stimulation

and negative for the hematopoietic lineage markers CD31, CD34, CD45 and HLA-DR after 6 passages (Fig. 1B). The viability of these cells was not appreciably affected when treating them with various concentrations of poly(I:C) (1 μg/ml to 20 μg/ml) for 4 h (Fig. 1C). These data indicate that TLR3-stimulated hMSCs maintain the typical characteristics of hMSCs.

IDO gene expression and chemokine expression in TLR3-stimulated hMSCs

To determine the appropriate time points, we performed mRNA and protein expression analysis in hMSCs treated for 0.5 h to 24 h with poly(I:C) at 10 μg/ml. The poly(I:C) treatment induced the expression of IDO (Fig. 1D) as well as chemokine (C-C motif) ligand 5 (CCL5) and chemokine (C-X-C motif) ligand 10 (CXCL10) (Fig. 1E and F). While the time profiles differed, induction was in all cases maximal or near-maximal at 4 hours. Based on these results, we used the 10 μg/ml concentration and the 4 h time point as the standard condition for TLR3 stimulation of hMSCs.

Differentially expressed genes of TLR3-stimulated hMSCs

To analyze the gene expression pattern in response to TLR3 stimulation, three independent samples (biological triplicates) of the entire transcriptomes of the control 7F3915 hMSCs and TLR3-stimulated 7F3915 hMSCs were profiled using RNA-sequencing. A total of 130 genes were differentially expressed, with 120 upregulated and 10 downregulated genes in the TLR3-stimulated versus control hMSCs. Of these differentially expressed genes (DEGs), the top 50 upregulated genes are listed in Fig. 2A. Interestingly, most of the top 50 genes were related to interferons and inflammation, namely, an interferon-induced protein with tetratricopeptide repeats 2 (IFIT2), CXCL8, IFIT3, interleukin 1 beta (IL1B) and 2'-5'-oligoadenylate synthetase-like (OASL) (Fig. 2A and Supplemental Table 1). Next, we performed functional annotation analysis for Gene Ontology (GO) based on the biological processes (BP) using DAVID Informatics Resources. The upregulated genes in the TLR3-stimulated hMSCs were determined to be involved in several BPs such as response to viruses, inflammation, and anti-apoptosis (Fig. 2B).

Furthermore, normalized RNA-seq read densities of inflammation-related (CXCL1 and CXCL8) and interferon-related genes (IFIT1 and IFIT2) were increased in TLR3-stimulated hMSCs (Fig. 2C). To validate the RNA-seq results, we confirmed the expression of the DEGs by qRT-PCR. Because viral dsRNA poly(I:C) was used to treat the hMSCs, we selected inflammation-related (CCL2, CXCL1, CXCL3, and CXCL8), and interferon-related (guanylate binding protein 1 (GBP1), GBP2, IFIT1 and IFIT2) genes for analysis. GBP1, GBP2, and IFIT1 mRNAs were upregulated at 4 h; CCL2, CXCL1, CXCL3, CXCL8, and IFIT2 mRNAs at 2 and 4 h (Fig. 2D).

To identify the degree to which the 7F3915 hMSCs genes that we detected were upregulated in 7F3674 and 127756 hMSCs, we further constructed RNA-seq dataset to compare the upregulated genes in 7F3915 hMSCs with that of 7F3674 and 127756 hMSCs. We compared the identified molecular signatures of the most highly upregulated genes that we found to be expressed in 7F3915 hMSCs to those of 7F3674 and 127756 hMSCs. Of top 30 upregulated genes in TLR3-stimulated 7F3915 hMSCs, 7F3674 and 127756 hMSCs shared 20 genes following TLR3 stimulation, suggesting substantial similarities in their transcriptional regulation among different donors hMSCs (Supplemental Fig. S1A, S1B). The following inflammation-related genes were upregulated all three different donors (7F3915, 7F3674 and 127756 hMSCs): cytokines/chemokines (CCL2, CXCL1, CXCL8, and CXCL8, etc.), interferon-related (IFIT1, IFIT2, IFIT3, IFI44, OASL, MX1, etc.). However, we found that most of the positive regulators of inflammation-related genes were more highly induced in 7F3915 hMSCs than in 7F3674 and 127756 hMSCs following TLR3 stimulation. We also observed 7F3674 and 127756 hMSCs also
had similarities in their physiological functions according to DAVID GO analysis, such as response to viruses, and immune response (Supplemental Fig. S1C).

**Immune cells (BV-2 microglial cell lines) do not acquire the hMSCs transcriptional signature**

To identify the degree to which the 7F3915 hMSCs genes that we detected were up-regulated in immune cell types, we analyzed our published RNA-seq dataset (40) to compare the up-regulated genes in TLR3-stimulated BV-2 microglial cell lines with that of 7F3915 hMSCs. Using Venn diagrams, and heat maps of BV-2 microglial cell lines and hMSCs transcriptome profiles, we found that hMSCs differed in their transcriptomes from BV-2 microglial cell lines (Supplemental Fig. S2A, S2C). We compared the identified molecular signatures of the top 100 up-regulated genes (Fold change ≥ 1.5, and \( P \leq 0.01 \)) between hMSCs and BV-2 microglial cell lines. In hMSCs, 71 genes were up-regulated that were not common to the BV-2 microglial cell lines. In contrast, in BV-2 microglial cell lines, also 71 genes were up-regulated that were not common to the TLR3-stimulated hMSCs (Supplemental Fig. S2A). The unique gene sets for hMSCs and BV-2 microglial cell lines are presented in Supplemental Fig. 2C. However, TLR3-stimulated hMSCs and BV-2 microglial cell lines also displayed similarities in their transcriptomes. The following inflammation-related genes were up-regulated only in hMSCs: cytokines/chemokines (CCL2, CCL20, CXCL1, CXCL3, CXCL6, CXCL8, IL7R, IL32, etc.), and interferon-related (GBP1, GBP4, IFI44, IFI44L, OAS1, OAS3, among others) (Supplemental Fig. S2C). Thus, hMSCs and BV-2 microglial cell lines maintain their molecular signature during inflammatory responses.

**Network analysis of the altered genes in TLR3-stimulated hMSCs**

Next, we used IPA software to identify the network of genes and related pathways that represent the interacting genes in TLR3-stimulated 7F3915 hMSCs. Networks 1 and 2 were among the networks of DEGs after 4 h of poly(I:C) treatment (Fig. 3, A and B). The genes in network 1 are known to be involved in cellular movement, immune cell trafficking, and inflammatory response; these genes formed a direct or indirect network with the NF-κB complex (Fig. 3A). The genes in network 2 are known to be involved in antimicrobial response, inflammatory response, and dermatological diseases and conditions; these genes formed a direct or indirect network with interferon regulatory factor 1 (IRF1) or the PI3K family (Fig. 3B). The top 10 canonical pathways that include DEGs in the TLR3-stimulated hMSCs are listed in Fig. 3C. Interestingly, the genes in canonical pathways related to inflammation encode pattern recognition receptors for bacteria and viruses, interferon signaling, and activation of IRF by the cytosolic pattern recognition receptor. To further characterize the TLR3-stimulated hMSCs, we determined the molecular and cellular functions of the upregulated and downregulated genes in TLR3-stimulated hMSCs. We found that the top 6 of these functions are related to the cellular movement, immunological disease, etc. (Fig. 3D).

**Differential expression of transcription factors in TLR3-stimulated hMSCs**

To better understand regulation, we focused on the transcription factors (TFs) among the DEGs in TLR3-stimulated 7F3915 hMSCs. Upregulated TFs notably included the NF-κB family, i.e. NF-κB1, NF-κB2, NF-κBIA, NF-κBIE, NF-κBIZ, REL, and RELB (Fig. 4A), as confirmed by qRT-PCR (Fig. 4E). Next, we used IPA software to identify the target genes that were directly or indirectly regulated by TFs in TLR3-stimulated hMSCs. Interestingly, we found that cytokines, chemokines and inflammation-related genes, including CXCL8, CXCL10, IL6, and IRF1, were regulated by TFs, including NF-κB1 and FOXO1 (Fig. 4B). Next, we used DAVID 6.8 software to identify functionally annotated processes of the FOXO1- and NF-κB1-regulated genes. We observed an enrichment of FOXO1 target genes associated with protein oligomerization and response to interleukin-1
FOXO1 controls hMSCs migration to TLR3 stimulation

(Fig. 4C). NF-κB1-regulated genes were determined to be related to the chemokine-mediated signaling pathway and chemotaxis (Fig. 4D). The FOXO1 and NF-κB1-regulated transcripts were determined to be associated with cytokine-mediated responses and immune responses. We analyzed the expression of several FOXO genes using real-time PCR and immunocytochemistry (Fig. 4F and 6B). While FOXO1 was significantly upregulated, the expression of FOXO3 and FOXO4 did not change after poly(I:C) treatment (Fig. 4F and Supplemental Fig. S3A, S3B). We also analyzed the expression of FOXO1, FOXO3, and FOXO4 in 7F3674 and 127756 hMSCs (Supplemental Fig. S4A and S5A). The MSCs from both donors showed up-regulated FOXO1 gene expression upon poly(I:C) treatment.

**Upregulation of cell migration-related genes in TLR3-stimulated hMSCs**

In the therapeutic application of stem cells, their migration is important for wound healing effects. Therefore, after functional analysis of the up- and down-regulated genes in TLR3-stimulated 7F3915 hMSCs, we focused on cellular movement, except for genes associated with the inflammatory response and infectious disease-related functions. Using IPA software, we found that 42 genes, including cytokine/chemokine genes, were associated with cell migration responses (Fig. 5A). The IPA software further revealed that the cell migration response-related genes are regulated by NF-κB1 and FOXO1 (Fig. 5B). Using the Pscan software tool, we analyzed TF motifs to identify cis-regulatory elements within the promoter regions in cell migration response-related genes. The binding sites of NF-κB1, RELA, FOXO1, and FOXO3 were significantly enriched in TLR3-stimulated hMSCs (Fig. 5C). Again, FOXO1 was found to be associated with the cell migration response.

Role of FOXO1 in the migratory response of TLR3-stimulated hMSCs

The foregoing bioinformatics analysis suggested an important role for FOXO1 in the migration response. In order to obtain direct experimental support for this notion, we treated the cells with the FOXO1 selective inhibitor AS1842856 (41, 42) and determined its effects on the migratory response. Using the WST1 assay, we first determined the highest non-toxic concentration of AS1842856. Viability was not affected at lower doses (1 and 5 μM) but was slightly decreased at higher doses (10, 20 μM) (Fig. 5D). Therefore, 5 μM was used in the following.

First, we measured the expression of selected cell migration-related genes (Fig. 5E). We found that AS1842856 significantly downregulated the expression of key cell migration-related genes, including CCL2, CCL5, CXCL1, CXCL8, CXCL10, ICAM1, IFIT2, IL6, MMP1, MMP3, NF-κB1, NF-κB2, SOD2, TNFSF10, and VCAM1 (Fig. 5E). By contrast, some of the genes regulated by NF-κB (ATF3, HMOX1, IFITM1, and PTGS2) did not show altered expression (Supplemental Fig. S6). We also analyzed the expression of cell migration-related genes in MSCs from 7F3674 and 127756 hMSCs. In both cell isolates, inhibition of FOXO1 regulated the expression of cell migration-related genes (Supplemental Fig. S4B and Supplemental Fig. S5B). Next, we investigated whether FOXO1 depletion affects key cell migration-related genes. hMSCs were treated with three different siRNA (si5257, si5258, and si5259), and si5258 led to significant depletion of FOXO1 expression compared to the negative control group (NC: scrambled siRNA) (Supplemental Fig. S7A). Furthermore, none of these FOXO1 siRNAs were able to inhibit positive control GAPDH (Supplemental Fig. S7A). We also used positive control siRNA (GAPDH siRNA) to evaluate the methods of transfection and found that the positive control siRNA also had significant depletion of GAPDH, but not FOXO1 (Supplemental Fig. S7B). Therefore, we used si5258 in the subsequent experiments. Using siRNA analysis we found that key cell migration-related genes were increased after poly(I:C) treatment and FOXO1 depletion cells led to significant reduction in key cell migration-related genes (Fig. 5F). However, some of the migration...
related genes, including CXCL1, NF-κB1, NF-κB2, and SOD2 did not show low expression level (data not shown).

Furthermore, we performed chromatin immunoprecipitation (ChIP) experiments in order to determine FOXO1 occupancy in target genes promoter regions. FOXO1 directly bound to CCL2, CXCL8, and ICAM1 promoter regions (Fig. 5G). These findings suggest that FOXO1 might be involved in the regulation of cell migration in TLR3-stimulated hMSCs.

Second, we performed a wound healing assay with control cells, TLR3-stimulated hMSCs, AS1842856-treated unstimulated cells, and AS1842856-treated TLR3-stimulated cells. These cells, including AS1842856-treated TLR3-stimulated cells, displayed similar spindle-shaped fibroblastic morphology (Fig. 6A). No morphological effects were visible in the TLR3-stimulated cells treated with AS1842856. To determine the efficiency of AS1842856, we analyzed FOXO1 mRNA and protein levels using qRT-PCR and immunocytochemistry for 10 and 30 minutes. Immunofluorescence experiments revealed that AS1842856 treatment prevented the increased translocation of FOXO1 in TLR3-stimulated hMSCs (Fig. 6B). Furthermore, the qRT-PCR results revealed that in the TLR3-stimulated hMSCs, AS1842856 significantly downregulated the expression of FOXO1 but not FOXO3 and FOXO4 (Fig. 6 C). In addition, we performed a wound-healing assay to investigate the migration effects in TLR3-stimulated cells. Poly(I:C) treatment significantly increased the number of hMSCs that migrated into a wound field for 24 h. AS1842856 treatment in TLR3-stimulated hMSCs significantly decreased the number of cells that migrated into a wound field compared to the control cells (Fig. 6D). We also performed transwell migration assay in order to determine hMSCs migration in response to the AS1842856. We found that AS1842856 significantly suppressed the migration of 7F3674 and 127756 hMSCs towards TLR3 stimulation (Supplemental Fig. S8A, S8B). Taken together, these data strongly suggest that FOXO1 is involved in the regulation of cell migration responses in TLR3-stimulated hMSCs.

DISCUSSION

In addition to their differentiation potentials, the immunomodulatory properties of MSCs create exciting possibilities for therapies that aim to repair damaged tissues (12,30). MSCs inhibit the proliferation of immune cells, including T cells, macrophages, natural killer cells and dendritic cells (4,7,28). MSCs are widely investigated for their use in the treatment of inflammatory diseases, such as graft-versus-host disease, collagen-induced arthritis, experimental autoimmune encephalomyelitis, type 1 diabetes mellitus disease and inflammatory bowel disease (43).

MSCs can be isolated and expanded ex vivo before being administered to patients (14,44-46). In many studies, researchers used the expansion phase for pretreatments hoped to enhance therapeutic effects such as immunomodulation, transdifferentiation, and cell migration (11,47-49). In this respect, Toll-like receptors (TLRs) are promising targets, since they are known to modulate immune responses in inflammation-associated conditions such as infection and tissue injury (28,33,50). Of the various TLRs, the present study has focused on TLR3. Activation of TLR3 is known to induce the expression of cytokines and chemokines that repress T cell proliferation, exert various immunosuppressive effects, and stimulate cell migration (2,24,51). In agreement with this notion, TLR3-stimulated MSCs (poly(I:C)-treated MSCs) exhibit enhanced immunosuppressive properties. For example, they inhibit T cell proliferation and reduce intestinal inflammation in dextran sulfate sodium-induced acute colitis (52). Poly(I:C) treatment also improves the immunosuppression effects of MSCs in septic mice (53) and ameliorates myocardial fibrosis, reduces apoptosis, and enhances cardiomyocyte regeneration in hamsters (54). Preconditioning of human umbilical cord-derived MSC (hUC-MSCs) with poly(I:C) protects against mortality in trinitrobenzene sulfonate-induced colitis in mice (55). These examples illustrate that it is of medical interest to clarify the molecular mechanisms triggered by TLR3 stimulation in
FOXO1 controls hMSCs migration to TLR3 stimulation

hMSCs.

The approach taken in the present study was to identify, by RNA-seq technology, the DEGs of TLR3-stimulated versus control hMSCs. It has been known that activation of TLR3 promotes IDO1 production in MSCs, which thereby repress T cell proliferation and enhance immunosuppression (2). TLR3 stimulation activates the immunosuppressive effects by indirectly inducing the expression of IDO1 (23,33,39). The present study more comprehensively identified DEGs of TLR3-stimulated hMSCs that are associated with cell migration and the inflammatory response. These include cytokine and chemokine genes (CCL2, CCL5, CCL20, CXCL1, CXCL2, CXCL6, CXCL8, CXCL10, and CXCL11), interleukin-related genes (IL1A, IL1B, IL6, and IL32) and interferon-stimulating genes (GBP1, GBP4, IFIT1, IFIT2, IFIT3, IRF1, ISG15, MX1, OAS1, OAS2, and OAS3) (Fig. 2A).

Of the above list, CXCL1, CXCL8, and CCL2 are well-studied examples of chemokines and cytokines involved in migration and chemotaxis (56). For example, Pu et al. reported that the upregulation of CXCL1 and CXCL8 promotes chemotaxis and regulates adiponectin-induced chemotaxis in a human jaw bone marrow MSCs (57). CXCL1 induces chemotaxis and stimulates wound repair in a scratch assay using bone marrow-derived mesenchymal cells (BM-MCs). In addition, an anti-CXCL1 antibody decreased CXCL1-stimulated migration activity in BM-MCs (58). Furthermore, CCL2 promotes the migration of SG2 cells, a TGF-β-responsive MSC line (59). MSC-derived CCL2 increases the migration of immune cells such as monocytes, while an anti-CCL2 antibody counteracts their migration (60). CCL2 is also important for the mediation of rat BM-MSC migration towards gliomas (61). Altogether, these literature results are consistent with our data that show increased cell migration and chemotaxis in association with the induction of cytokines and chemokines in TLR3-stimulated hMSCs (Fig. 2A-E).

TLR3 also activated inflammatory responses through the upregulation of infectious disease- and inflammatory response-related genes. In IPA network analysis, the infectious disease network was highly enriched with interferon-related genes (IFI6, IFIH1, IFIT1, IFNAR2, IRF1, IRF7, ISG15, and MX1) (Fig. 3B) that are regulated by the NF-κB family (Fig. 4B). TLR signaling leads to activation of downstream effectors, such as NF-κB, MAPK, and IRFs. Amongst these, NF-κB plays a key role in the induction of inflammatory cytokine genes and the induction of chemotaxis or cell migration (62,63). In our study, TLR3 stimulation induced the expression of the NF-κB family, such as NF-κB1, NF-κB2, NF-κBIA, NF-κBIE, NF-κBIZ, REL, and RELB (Fig. 4A, E). Thus, in agreement with the literature, our data indicate that NF-κB controls inflammatory response genes, chemokine-related pathways, chemotaxis, and cell migration in TLR3-stimulated hMSCs.

To further analyze transcriptional regulation downstream of TLR3 stimulation, we performed transcription factor (TF) binding motif analysis. We found that the promoters of cell migration-related genes were enriched for putative binding sites of FOXO1, FOXO3, NF-κB1, and RELA. IPA software then identified the NF-κB1 and FOXO1 target genes that are involved in similar biological processes including immune response and cytokine-related pathways (Fig. 5B). In a previous study, it was shown that FOXO1 regulates immune responses by protecting hematopoietic stem cells against oxidative stress (64). FOXO1 induces cell migration through TGFβ1 in keratinocytes, and knockdown of FOXO1 inhibits keratinocyte migration (65). Taken together, these results suggest that FOXO1 and NF-κB1 have an important role in TLR3-mediated cell migration as well as immune responses of hMSCs.

Amongst the DEGs identified by RNA-seq, the genes of adhesion molecules and matrix metallopeptidases (MMPs) were significantly upregulated in TLR3-stimulated hMSCs. Adhesion molecules, such as ICAM1 and VCAM1, play a critical role in MSCs migration (66) and mediate TNF-α-stimulated MSCs migration (67). Likewise, MMPs play an essential role in cell migration (68). Ho et al. reported that migration properties parallel the activation and inhibition of MMP1 (69). In the present study, MMPs and
FOXO1 controls hMSCs migration to TLR3 stimulation

adhesion molecules such as ICAM1, MMP1, MMP3, and VCAM1 were significantly upregulated by TLR3 stimulation. Furthermore, the expression of cell migration-related cytokine and chemokine genes (CCL2, CCL5, CXCL1, CXCL8, and CXCL10), an interferon-related gene (IFIT2), NF-κB family genes (NF-κB1 and NF-κB2), and TNF-related genes (TNFSF10) were significantly up-regulated in TLR3-stimulated hMSCs. Importantly, treatment with AS1842856, a highly specific FOXO1 inhibitor, blunted these effects of TLR3 stimulation on gene expression (Fig. 5E) as well as the associated cell migration response. Interestingly, many studies researchers used AS1842856 for the specific inhibition of FOXO1, since they are known to affecting only FOXO1 transcription-activating domain (41, 42, 70). Nevertheless, further experimental validation using knockouts or overexpression models are warranted to determine the role of FOXO1 in MSCs. Taken together, our results demonstrate that FOXO1 is important for cell migration and the regulation of cell migration-related genes in TLR3-stimulated hMSCs.

Overall, our RNA-seq data reveal the transcriptional landscape of TLR3-stimulated hMSCs, reflecting the altered gene expression and modulation of cell responses by external stimulation. Based on these data, we have used gene expression profiling, gene clustering, pathway analysis, and TF motif analysis to generate information about the molecular mechanisms that underly therapeutic effects such as immunomodulation and cell migration. Future analysis of histone modifications, TF binding, and non-coding RNA expression will provide further insights into the regulatory systems that control the therapeutically relevant cell migration-related gene expression in TLR3-stimulated hMSCs.

CONCLUSION

Our comparative analysis of global transcriptomes revealed the altered expression of inflammation- and cell migration-related genes in TLR3-stimulated versus non-stimulated hMSCs. Bioinformatics approaches identified NF-κB and FOXO1 as potential regulators of the migratory response of TLR3-stimulated hMSCs. In support, direct inhibition of FOXO1 significantly down-regulated the expression of cell migration-related genes and cell migration responses. Together, these findings provide a better understanding of the molecular mechanisms that underlie the TLR3-mediated migration response of hMSCs.

EXPERIMENTAL PROCEDURES

Cell culture and stimulation of hMSCs

Human bone marrow-derived mesenchymal stromal cells (hBM-MSCs) were derived from donors 7F3915 (21-year-old female), 7F3674 (22-year-old female), and 127756 (43-year-old male). These cells were purchased from Lonza (Walkersville, MD) and cultured in low glucose Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Waltham, MA) and penicillin (100 U/ml)/streptomycin (100 mg/ml) (Gibco, Waltham, MA). Most experiments were performed using hBM-MSCs at passage 6. The cells were maintained in a humidified incubator with 95% air and a 5% CO2 atmosphere at 37°C and FOXO1 inhibitor AS1842856 (5-amino-7-(cyclohexylamino)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid) (41), were purchased from Merck Millipore, Billerica, MA. The cells were treated with the TLR3 agonist poly(I:C) (10 µg/ml, Sigma-Aldrich, St. Louis, MO), and AS1842856 for the specified concentrations under normal culture conditions. At 70-80% confluency, they were treated for 4 h with fresh growth medium (control) or growth medium containing poly (I:C) (TLR3-stimulated MSCs), and AS1842856.

Characterization of hMSCs using flow cytometry analysis

Flow cytometry analysis was performed as previously described (63). hMSCs were harvested using trypsin and incubated for 30 min
FOXO1 controls hMSCs migration to TLR3 stimulation

with fluorescein isothiocyanate (FITC)-conjugated antibodies against CD105, HLA-DR, or CD29 (all from Serotec Ltd., Oxford, UK), CD44 (DakoCytomation, Glostrup, Denmark), or with phycoerythrin (PE)-conjugated antibodies against CD34 (Serotec Ltd., Oxford, UK), CD45 (DakoCytomation, Glostrup, Denmark), CD31 (DakoCytomation, Glostrup, Denmark), CD73 (BD Pharmingen, San Diego, CA), or CD90 (BD Pharmingen, San Diego, CA). Control cells were prepared with FITC- and PE-mouse isotype control antibodies (Serotec Ltd., Oxford, UK). The stained cells were analyzed with a FACS Aria II (BD Bioscience, San Diego, CA) flow cytometer.

Chemokine measurements with enzyme-linked immunosorbent assay (ELISA)

Human MSCs were treated with poly(I:C) (10 µg/ml) for 4 h. After treatment, the concentration of the inflammatory chemokines CCL5 and CXCL10 in cell culture supernatants were determined using human CCL5 and CXCL10 ELISA kits (KOMABIOTECH, Seoul, Korea) according to the manufacturer’s instructions. The data represent three independent experiments (n=3).

Cell viability assay

The cell viability assay was performed using a tetrazolium salt colorimetric assay with PreMixWST-1 according to the manufacturer’s instructions (Takara, Shiga, Japan). The cells were seeded at a density of 1 X10^4 per well in 96-well plates and incubated from 0 h to 96 h. After this incubation, PreMixWST1 was added to each well, and the plate was incubated for an additional 4 h. Finally, the absorbance of the microplate at 450 nm was measured. The data represent three independent experiments (n=3).

Transcriptome analysis using RNA-sequencing (RNA-seq)

RNA-seq was performed as previously described (71). Total RNA was extracted using RNAiso Plus (Takara, Shiga, Japan) and the QIAGEN RNeasy® Mini Kit (QIAGEN, Hilden, Germany). Ribosomal RNA (rRNA) was depleted by the RiboMinus Eukaryote kit (Invitrogen, Carlsbad, CA). An RNA library was created by the NEBNext® Ultra™ directional RNA library preparation kit from Illumina® (New England BioLabs, Ipswich, MA). The RNA library sequencing was performed on the Illumina HiSeq2000 platform. Transcriptome sequencing was performed on independent RNA samples from the control 7F3915 hMSCs (3 samples), TLR3-stimulated 7F3915 hMSCs (3 samples), control 7F3674 hMSCs (3 samples), TLR3-stimulated 7F3674 hMSCs (3 samples), control 127756 hMSCs (3 samples), and TLR3-stimulated 127756 hMSCs (3 samples) in biological triplicate.

To analyze the differentially expressed genes, FASTQ files from RNA-seq were clipped, trimmed of adapters, and reads of low-quality were removed using Trimmomatic (72). These FASTQ files were aligned using the STAR (version 2.5.1) aligner software (73) with the UCSC hg19 reference. We analyzed the differential gene expression using DESeq2 with the default parameters (74). Genes identified by RNA-seq that had an absolute log2-fold change larger than 0.7 (log2-fold change ≥ 0.7 and log2-fold change ≤ –0.7, P-value < 0.01) were selected as being differentially expressed genes (DEGs) in TLR3-stimulated hMSCs. The acquired data were deposited in the Gene Expression Omnibus database under dataset accession no. GSE97724.

Analysis of the networks and pathways

The RNA-seq dataset was mapped using Ingenuity's Knowledge Base IPA (Ingenuity System, Mountain View, CA) to analyze the networks and pathways. RNA-seq data were cutoff at the fold-change (≥0.7 log2-fold change, P-value ≤ 0.01) in TLR3-stimulated hMSCs.

Functional annotation

To determine functional annotations, gene ontology analysis was performed using DAVID
FOXO1 controls hMSCs migration to TLR3 stimulation

(Database for Annotation, Visualization, and Integrated Discovery) version 6.8 (75). The gene ontology was examined using a modified Fisher’s exact $P$-value in the DAVID program. Annotation categories with $P$-values less than 0.001 were considered to be enriched.

Transcription factor binding motif enrichment analysis

GenBank accession numbers were input into the PsCan software to analyze transcription factor binding motifs. The JASPAR 2016 database of TF binding sequences of the promoter regions ranged from 1000 base pair (bp) downstream of the transcription start site to $+0$ bp (76). The web-based software PsCan was used for analysis of the transcription factor binding motifs using the GenBank accession number (77).

Gene expression analysis using quantitative real-time RT-PCR

Total RNA was extracted from hMSCs using RNAiso Plus (Takara, Shiga, Japan) according to the manufacturer’s instructions. The cDNA was generated by PrimeScript reverse transcriptase (Takara, Shiga, Japan). The synthesized cDNA was amplified using gene-specific primers (Table 1). The primers were designed by Primer Bank (http://pga.mgh.harvard.edu/primerbank/index.html). Real-time PCR was performed with SYBR Premix Ex Taq™ II (Takara, Shiga, Japan). Glyceraldehyde-3-phosphate dehydrogenase was used as the internal control. The data represent three independent experiments (n=3). After performing real-time PCR, the results were analyzed using the critical threshold ($\Delta CT$) and the comparative critical threshold ($\Delta\Delta CT$) methods in the ABI 7500 software with the NormFinder and geNorm PLUS algorithms.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as previously described (63). Briefly, chromatin from $2 \times 10^7$ cells was used for ChIP assay. The cells were immunoprecipitated with antibodies against FOXO1 (Abcam, Cambridge; ab39670) and normal rabbit IgG (Santa Cruz Biotechnology, Inc., Dallas, TX; sc#2025) used as a control with Dynabeads Protein A beads (Invitrogen, Waltham, MA). The immunoprecipitated DNA was analyzed by real-time quantitative PCR, and the resulting levels were normalized to the amounts of input DNA. The data represent three independent experiments (n=3). Primers used for ChIP-PCR are listed in Table 2.

In vitro wound healing assay

The wound healing assay was performed as previously described (63). Briefly, the cells were seeded onto Ibidi culture inserts (Ibidi, Martinsried, Germany) and incubated for 24 h. After incubation, the cells were treated with either poly(I:C) or FOXO1 inhibitor AS1842856 for 4 h. The insert was removed and incubated in growth medium for 24 h. The cells were imaged by an inverted microscope (Leica, Wetzlar, Germany).

Migration assay

Transwell migration assays were performed using Fluoroblock migration plates as described previously (78). Briefly, hMSCs were loaded at density $5 \times 10^4$ cells with Calcein AM (Thermo fisher C3100MP), washed prior to seeding hMSCs in the upper chamber of the tissue culture insert. After 24 h in culture, the hMSCs were stimulated for 4 h with poly(I:C) (10 $\mu$g/ml) in the presence or absence of FOXO1 inhibitor AS1842856 (5 $\mu$M). Poly(I:C) and AS1842856 were added to the lower chamber to stimulate migration. Then hMSCs were rinsed and the medium was replaced. After 24 h incubation, the fluorescence of migrating hMSCs were measured using an inverted fluorescence live cell imaging system (Leica, Wetzlar, Germany).

Immunocytochemistry

The cells were incubated on coverslips in 4-well plates. After 24 h, the cells were treated with either poly(I:C) or FOXO1 inhibitor, washed with
PBS and then fixed with 4% paraformaldehyde. Next, the cells were incubated with cold methanol for 5 min and blocking solution (5% BSA in PBS) for 1 h. The cells were then incubated with the primary antibody anti-rabbit FOXO1 (1:200, Abcam, Cambridge, UK) and the secondary antibody donkey anti-rabbit IgG (Jackson Laboratory, West Grove, PA). After incubation with the antibodies, the cells were washed with PBS, mounted with 4’, 6-diamidino-2-phenylindole (DAPI) mounting solution (Vectashield, Vector Laboratories, Burlingame, CA) and imaged by microscopy (Zeiss, Oberkochen, Germany). The percentage of total fluorescence intensity ratio of nuclear vs. cytoplasmic area was determined and quantified with the Intensity Ratio Nuclei Cytoplasm macro from ImageJ (http://rsb.info.nih.gov/ij/download.html) software, using DAPI staining to define the nucleus area available from National Institutes of Health as described (79).

Knockdown of FOXO1 gene expression by siRNA treatment

Oligonucleotide specific for FOXO1 (ID # s5257, s5258, and s5259) was from Thermo Fisher Scientific (Waltham, MA). MSCs cells were transfected with FOXO1 siRNA constructs following the Silencer Select siRNA transfection protocol with non-targeting siRNAs (Ambion Applied Biosystems, Waltham, MA). FOXO1 siRNA was used at concentrations of 5 nM for 48 hr using DMEM medium.

Western blotting

The extraction of protein from the cells was performed using RIPA buffer [1% Triton X-100 in 50 mM phosphate buffer (pH 7.4)] containing a complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). The extracted protein was separated on SDS polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Schleicher & Schuell Bioscience, Inc., Keene, NH). The Western blot analysis was performed using anti-FOXO1 (Abcam, Milton, Cambridge, UK; ab39670) and anti-β-actin (Sigma-Aldrich, St. Louis, MO; A-5316) antibodies. β-actin was used as an internal control.

Statistical analysis

Data were analyzed as the means ± the standard deviation of the mean (SD). Data were analyzed using the SPSS 17.0 program (SPSS Inc., Chicago, IL) using a one-way ANOVA, followed by the Tukey’s honestly significant difference (HSD) post hoc test. P-values < 0.05 were considered significant.

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Competing of interests:

The authors declare no conflicts of interest.

Author’s contributions:

S. H. K. designed and performed experiments, analyzed and interpreted the data, and prepared the manuscript; A. D. designed, analyzed next-generation sequencing and bioinformatics data, interpreted the data, prepared, critically revised, and edited the manuscript; J. C. C. analyzed next-generation sequencing
and bioinformatics data; K. H. K. analyzed and interpreted the data; M. R. C. designed experiments, analyzed and interpreted the data; H. C. performed experiments. B. B. designed experiments, analyzed and interpreted the data; Y. S. L. analyzed next-generation sequencing and bioinformatics data; K. H. J. designed experiments, financial support, analyzed, critically revised, interpreted the data, prepared and edited the manuscript; Y. G. C. designed experiments, financial support, analyzed and interpreted next-generation sequencing and bioinformatics data, final approval of manuscript, edited the manuscript.

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FOXO1 controls hMSCs migration to TLR3 stimulation

The abbreviations used are: BP, biological processes; CCL2, chemokine (C-C motif) ligand; ChIP, chromatin immunoprecipitation; CXCL, chemokine (C-X-C motif) ligand; DEGs, differentially expressed genes; eRNA, enhancer RNA; FOXO1, forkhead box protein O1; GO, gene ontology; hMSCs, human mesenchymal stromal cells; IDO, indoleamine 2,3-dioxygenase; PGE2, prostaglandin E2; poly(I:C), polyinosinic:polycytidylic acid; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RNA-seq, RNA-sequencing; TF, transcription factors; TLR, Toll-like receptor;

Table 1: List of primers used in qRT-PCR studies

| Gene symbol | Forward Sequence (5' -> 3') | Reverse Sequence (5' -> 3') |
|-------------|-----------------------------|-----------------------------|
| CCL2        | CAGCCAGATGCAATCAATGCC        | TGGAATCCTGAACCCACCTTCT      |
| CCL5        | CAGCACGTGGGACCTGCAACA       | GGCAGTGGGCGGGCAATGTA        |
| CXCL1       | AGGCCAGGGGAAATGTATGTGC      | AGCCCCCTTGTCTAAGCAGCA       |
| GAPDH       | AAGGTCGGAGTCAACGGATT        | CTCTGGAGATGTTGAGTGAGG       |
| CXCL3       | CGCCCAAACCAGAAGCTCATAG     | GCTCCCCCCGTTAGTACATCTTT     |
| CXCL8       | CAAACTTTCCACCCCCAAAT       | CTCAGCCCTCTTACAAAAACT       |
| CXCL10      | GGAAGGTTAATGTTCATCATTCATAAGC| TAGTACCCCTTGGAGAGATGGGAAAG |
| FOXO1       | TCGTCATAATCTGCTCCCTACACA   | CGGCTTGCAATCTTACAAAA        |
| FOXO3       | CGGACAAACGGCTCATCTCT       | GGACCACATGAATCGACTAT        |
| FOXO4       | GGCTGCGGCGATCATAGAC        | GGCTGGTAGCGGATACCTGG        |
| GBP1        | AGCCCTACAAACTTGCGGAACAG    | TCTGGATTCGCCATACAGTGCG      |
| GBP2        | CTTAGTGCAATCTGAGCTCT       | GGTGCTGTTACGCACTTACAGC      |
| ICAM1       | ATGCCAGACATCTGTGC          | GGGGTCTCATGACCACCAAC        |
| IDO1        | GCCAGCTTCGAGAAAGAGTTG      | ATCCGAAACTGACGCTGCAA        |
| IFIT1       | TTGATGACGTAAATGCTGGAATGCTG | CAGGTCACAGACTCCTCAGC        |
| IFIT2       | AAGCACCCTCAAGGGCAAAAAC     | TCGGCCATGTGATAGCAGC         |
| IL6         | CAGGAGAAGATTCCAAAGAT       | CTCTGTATGACAGCTCTTT         |
| MMP1        | CTCTGGAGAATGCTCACACCTCT    | TGGTGGTACACCTTTTACTCTTC     |
| MMP3        | CGGTTCGGCTGTCTCAAGG        | GCACAAATGGCTGCTCTTT         |
| NFKB1       | GAAGCAGCAATGACAGAGGC       | GCTTGGCGAGATTAGCTTT         |
FOXO1 controls hMSCs migration to TLR3 stimulation

| Gene symbol | Forward Sequence (5' -> 3') | Reverse Sequence (5' -> 3') |
|-------------|-----------------------------|-----------------------------|
| NFKB2       | ATGGAGAGTTGCTACAACCCA       | CTGTTCCACGATCACCAGGTA       |
| NFKBIA      | CTCCGAGACTTTTCGAGGAAATAC    | GCCATTGTAGTTGGTAGCCTTCA     |
| NFKBIE      | TCTGGCATTGAGTCTCTGCG        | AGGAGCCATAGGTTGGAATCAG      |
| NFKBIZ      | AGAGGCCCCTTTTCAAGGTGT       | TCCATCAGACAACGAATCGGG       |
| RELB        | CCATTGAGCGGAAGATTCAGT       | CTGCTGGTCGCCGATATGAGG       |
| SOD2        | GGACACTTACAAATTGCTGCTTGT    | AGTAAGCGTGCTCCCAACACAT      |
| TNFSF10     | TGCGTGCTGATCGTGATCTTTC      | GCTCGTTGGTAAAGTACACGTA      |

Table 2: List of primers used in ChIP-PCR studies

| Gene symbol | Forward Sequence (5' -> 3') | Reverse Sequence (5' -> 3') |
|-------------|-----------------------------|-----------------------------|
| CCL2 R-1    | ATTTCTTCTGCAGCACTACAGTC    | GCCTAGCAGAAGCTGTAAGGATT     |
| CCL2 R-2    | CATCTGTGGTCAGTCTGCGC       | TCAAGCAGAGGAGGATCT          |
| CXCL8 R-1   | TAGTATGCCCTCTAAAGAGCAGT    | GATGGTAAGATAAAGCCAGCC       |
| CXCL8 R-2   | TGATGGCAGTCTAGGTTGCC       | CTTATGGAAGTGCTCGGTGG        |
| ICAM1 R-1   | ATCACGCAGCTTCCCTCTCTTT     | GTCCACACCTAGCTGACACG        |
| ICAM1 R-2   | CGCGGTTAGACCCGTGATTC       | TTCCCCCTGCCGAGAAATGC        |
FOXO1 controls hMSCs migration to TLR3 stimulation

FIGURES

Figure 1. The characteristics of TLR3-stimulated hMSCs. (A) No morphological changes were evident in control vs. TLR3-stimulated hMSCs. Original magnification: X100. (B) Immunophenotypes revealed by flow cytometry. The control and TLR3-stimulated hMSCs were positive for expression of the antigens CD29, CD44, CD73, and CD105. (C) Cell viability was determined by the WST1 assay. hMSCs were cultured for 1, 2, and 3 days. Cell viability is represented by the relative absorbance at 450 nm. (D) Quantitative real-time PCR analysis revealed that IDO1 expression was induced by TLR3 stimulation. The values are means ± SD of triplicate wells. **P < 0.005. (E, F) ELISA results showing the release of CCL5 and CXCL10 upon TLR3 stimulation of hMSCs. The values are means ± SD of triplicate wells. **P < 0.005.
**Figure 2. Differentially expressed genes in TLR3-stimulated 7F3915 hMSCs.** (A) Heat map of the top 50 upregulated genes in TLR3-stimulated vs control hMSCs ($P$-value < 0.05, log2-fold change ≥ 0.8). Each row indicates the relative expression of genes and each column indicates the expression level of each sample. Each condition was performed in biological triplicate (n=3). (B) Gene Ontology analysis was performed for the upregulated genes in the TLR3-stimulated hMSCs. (C) The UCSC genome browser images showed normalized RNA-seq read densities in the control and TLR3-stimulated hMSCs. (D) Validation of the differentially expressed genes by qRT-PCR in TLR3-stimulated hMSCs. The values are means ± SD of triplicate wells. ** $P < 0.005$. The data represent three independent experiments.
Figure 3. IPA-based network analysis of TLR3-stimulated hMSCs. (A, B) Ingenuity® Bioinformatics pathway analysis of the gene networks in TLR3-stimulated hMSCs. (C) The most highly represented canonical pathways for differentially expressed genes in TLR3-stimulated hMSCs. (D) The most highly represented molecular and cellular functions in TLR3-stimulated hMSCs.
FOXO1 controls hMSCs migration to TLR3 stimulation

Figure 4. Transcription factor families in TLR3-stimulated hMSCs. (A) Heat map of the differential expression of the NF-κB family. (B) The positive regulators of NF-κB and FOXO1 identified by using the IPA molecule activity predictor. (C, D) Gene ontology analysis using DAVID in TLR3-induced NF-κB and FOXO1 target genes. (E, F) Confirmation of the expression levels of the differentially expressed transcription factors NF-κB and FOXO using qRT-PCR. The values are the means ± SD of triplicate wells. **P < 0.005.
Figure 5. Regulation of the cell migration responses in TLR3-stimulated hMSCs. (A) Heat map representing the migration response-related genes in TLR3-stimulated hMSCs compared with the controls ($P$-value < 0.05, log$_2$-fold change ≥ 0.8). (B) The migration response molecules were analyzed with the IPA molecule activity predictor. Shown are the migration response molecules regulated by NF-kB and FOXO1. (C) Enrichment of transcription factor motifs within promoters of migration-related genes. (D) Effects of the FOXO1 inhibitor AS1842856 on cell viability. A high concentration (20 μM) of AS1842856 inhibited cell proliferation in hMSCs. (E) Effects of FOXO1 inhibition on the expression of stimulated genes in TLR3-stimulated hMSCs. The values are the means ± SD of triplicate wells. **$P$ < 0.01 and ***$P$ < 0.005. (F) Representative western blot and real time RT-PCR quantification showing relative mRNA expression levels of FOXO1 in scrambled siRNA control (n = 3) and FOXO1 siRNA treated hMSCs (n = 3). Attenuation of FOXO1 significantly decreased key cell migration-related genes by quantitative reverse transcription-polymerase chain reaction. The values are the means ± SD of triplicate wells. *$P$ < 0.05, **$P$ < 0.01, and ***$P$ < 0.005. (G) ChIP assay to analyze the binding of FOXO1 to target genes. The ChIP-enriched samples were subjected to quantitative PCR with primers targeting sub-regions of the selected genes promoters. The graphs represent the mean values of enrichment relative to input DNA from three independent experiments.
**P** < 0.005.

Figure 6. FOXO1 regulated cell migration responses in TLR3-stimulated hMSCs. (A) Effects of the FOXO1 selective inhibitor AS1842856 on cellular morphology. Poly(I:C) and AS1842856 did not affect the cellular morphology. Original magnification: X100. (B) Effects of poly(I:C) and AS1842856 on the cellular localization of FOXO1 using immunofluorescence microscopy. Original magnification: X400. Cells with nuclear localization of FOXO1 was determined. Quantification of nuclear FOXO1 fluorescence signal intensity in cells after 10 minutes and 30 minutes post Poly(I:C) AS1842856, and Poly(I:C) + AS1842856 treatment. Statistical representation of percentage of total fluorescence intensity of FOXO1 in the nucleus (%N) and the cytoplasm (%C) calculated. (C) Expression of FOXO1, FOXO3, and FOXO4 in AS1842856-treated hMSCs. The values are the means ± SD of triplicate wells. **P** < 0.005. (D) The migration of hMSCs was determined using a wound scratch assay. Migrating hMSCs after 24 h in TLR3-stimulated or AS1842856-treated hMSCs. The number of hMSCs that migrated into the middle blank fields was assessed. The data represent three biologically independent experiments.
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