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# Macrophage migration inhibitory factor induces phosphorylation of Mdm2 mediated by phosphatidylinositol 3-kinase/Akt kinase: Role of this pathway in decidual cell survival

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ABSTRACT

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway has an anti-apoptotic effect through several downstream targets, which includes activation of the transformed mouse 3T3 cell double-minute 2 (Mdm2) protein, its translocation to the nucleus and degradation of the tumor suppressor p53. We show that Mif, the Macrophage Migration Inhibitory Factor, an important cytokine at the maternal fetal interface in several species, triggers phosphorylation of Mdm2 protein in a PI3K/Akt-dependent manner, thereby preventing apoptosis in cultured mouse decidual cells. Inhibition of Akt and PI3K suppresses the pathway. Mif treatment also changes the nuclear translocation of p53 and interferes with the apoptotic fate of these cells when challenged with reactive oxygen species. In conclusion, an important mechanism has been found underlying decidual cell survival through Akt signaling pathway activated by Mif, suggesting a role for this cytokine in decidual homeostasis and in the integrity of the maternal-fetal barrier that is essential for successful gestation.



## 1. Introduction

The serine/threonine kinase AKT (protein kinase B or PKB) is a proto-oncogene with pivotal regulatory roles in several cellular physiological and pathological processes [1]. The AKT cascade is activated by a plethora of receptor-associated signals and phosphatidylinositol 3-kinase (PI3K) [1,2]. The downstream cascades regulate functions of numerous substrates involved in the regulation of cell survival, cell cycle progression, migration and protein synthesis [1,2]. The biological functions of AKT include mechanisms that have been studied particularly in the control of T-lymphocyte trafficking, insulin signaling, glucose metabolism, regulation of neuronal function, modulation of transforming growth factor β (TGFβ) signaling, organization of nuclear proteins and some pathological conditions of cancer [1,3–6].

As a survival factor, AKT suppresses the intrinsic apoptotic cell death machinery using the Bcl2 family member Bad as substrate, along with caspase 9; and also by phosphorylating and regulating the activity of cell death genes, such as p53, Fas ligand (homotrimeric type II transmembrane protein) and certain cytokines [1]. AKT kinase activity also catalyzes Mdm2 phosphorylation (murine double minute 2), leading to its nuclear translocation and the degradation of p53, a key factor inhibiting the apoptotic cascade [1,7]. Mdm2 regulates p53 activity through degradation and transcriptional restraint by ubiquitination and repression, respectively, of p53 transcriptional activity [7].

The maternal fetal interface is a complex anatomical structure where maternal and fetal components are mutually exposed. This

*Abbreviations:* MIF, Human macrophage migration inhibitory factor; Mif, mouse macrophage migration inhibitory factor; mrMif, mouse recombinant macrophage migration inhibitory factor; PI3K, phosphatidylinositol 3-Kinase; Mdm2, transformed mouse 3T3 cell double-minute 2; Akt, protein kinase B; LY or LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one); WT, Wortmannin; TRIC, triciribine; DAPI, 4, 6-diamidino-2-phenylindole.

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functional edge between mother and fetus is quite dynamic. It includes a temporal and spatially determined relationship between chorion/trophoblast cells and maternal blood, uterine luminal and glandular epithelium and mesenchyme-derived cells represented by decidual cells, leukocytes, endothelial cells and muscle cells. This interface constitutes a unique milieu, in which numerous regulatory molecules adjust the lodgment of the embryo/fetus within the uterine stroma and adapt the needs of both organisms for successful coexistence [8]. The expression of these molecules that includes cytokines, chemokines, growth factors, transcription factors, prostaglandins, adhesion molecules and others, seems to define specific patterns along the different stages of gestation [9,10].

During initial steps of pregnancy at this interface, a Th1 pro-inflammatory profile is prevalent, with expression of cytokines including IL (interleukin)-6, LIF (leukemia inhibitory factor), IL-8, IFN (interferon gamma)-γ and TNF (tumor necrosis factor)-α [8,11]. These cytokines are thought to recruit immune cells to the decidua to participate in the immunoregulation of tolerogenic mechanisms. Although it is an essential part of the adaptive gestational microenvironment, this inflammatory cytokine repertoire may also be involved in injury-induced mechanisms, in part due to the enhancement of the production of other cytokines and chemokines [8]. Analysis of the direct effect of IFNγ shows that this cytokine induces apoptosis dose and time dependently in cultured decidual cells [12].

Pro-inflammatory cytokine, the macrophage migration inhibitory factor (MIF), has also been identified at the maternal fetal interface in different species [13–16]. It is primarily an inhibitor of the random cellular migration from the peritoneal exudate. However, it can mediate many other biological activities such as activation of innate immunity, regulation of adaptive responses, expression of Toll-like receptor 4 (TLR-4), antagonism of glucocorticoids, and suppression of apoptosis induced and dependent on p53 [16,17].

The biological MIF activity is predominantly associated with its binding to the receptor/co-receptor complex CD74 and CD44, which can result in activation of different signaling pathways [18]. CD44 is part of the CD74-associated receptor complex in so far its intracytoplasmic domain is required for MIF signal transduction [18]. When only CD74 is expressed at the cellular surface, MIF can still signal through a mechanism that involves the endocytosis of the cytokine/receptor complex, recruitment of beta-arrestin 1 and activation of signal molecules that include Raf, MEK and pERK [19]. MIF signaling can also be mediated by its receptors, CXCR2 and CXCR4, that might be associated with CD74 [20] and, at least in part, by endosomal signaling mechanisms in a CD74/CXCR4-dependent pathway [21].

We have previously shown that murine *Mif* is expressed at the maternal fetal interface, with prevalent expression in trophoblast during early pregnancy, whereas expression of *CD74* and *CD44* receptors was detected in decidual cells [16,22]. We show here that Mif activates the PIK3/Akt signaling pathway in decidual cells, resulting in Mdm2 phosphorylation. In parallel, Mif reduces apoptosis in hydrogen peroxide ($H_2O_2$) treated decidual cells. We propose that Mif promotes decidual cell survival at the maternal fetal interface, thus contributing to local homeostasis and gestational success.

## 2. Material and methods

### 2.1. Animals

*Mus musculus domesticus*, CD-1 mice, aged 3–4 months were housed in the animal-care facility at the Institute of Biomedical Sciences at the University of São Paulo (Brazil) with water and pelleted diet *ad libidum* and on a 12 h:12 h light/dark cycle. Females were caged overnight with males (1:1) and successful allogeneic mating was checked daily for the presence of vaginal plugs. The morning a plug was found was designated as the first half day of pregnancy (gd 0.5). In all 165 pregnant females were used for the experiments. To analyze the maternal-placental interface, females at gd 7.5 were used. Procedures and animal handling were done in accordance with the guidelines provided by the Brazilian Society for Laboratory Animal Science (SBCAL), being certified by the Ethical Committee for Animal Use (CEUA) at the Institute of Biomedical Sciences, of the University of São Paulo.

### 2.2. Experimental design

Mesometrial area was isolated from implantation sites on gd 7.5 and decidual primary cultures were prepared. From the 165 pregnant females, around 1950 implantation sites were obtained and used to provide approximately 650 decidual culture plates (3:1). The number of cultures used in each experiment is detailed in the subsequent items of the methodology description. Cell viability, proliferation, apoptosis and necrosis, and protein concentration of phosphorylated Akt and Mdm2 were measured in cultures supplemented or not with: recombinant mouse mrMif (R&D Systems Inc., Minneapolis, MN, USA), inhibitors of phosphatidylinositol 3 kinase (PI3K): LY294002 (LY, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) and Wortmannin (WT) (Merck KGaA, Darmstadt, Germany) isolated or in association, inhibitor of AKT1/2/3 phosphorylation (triciribine, Sigma Chemical Co., St Louis, USA) and antibody against CD74 for receptor immunoneutralization. Experiments also involved $H_2O_2$ as an inducer of cell death. Nuclear translocation of p53 was evaluated by immunofluorescence. Samples from 3 independent experiments were assayed in triplicate. Decidual cells were examined morphologically, and for the purity of the isolated population through expression of specific cell markers (cytokeratin for epithelial cells; vimentin for mesenchymal-derived cells; alpha-2-macroglobulin and desmin for decidual cells [23–27]).

### 2.3. Preparing decidual cells

Implantation sites from gd 7.5 were used as source of decidual cells for culture according to Borbely et al. [12]. Briefly, on the morning of gd 7.5, the pregnant females were killed by cervical dislocation and their uteri immediately removed and kept in sterile cold PBS. Mesometrial decidua was isolated from other maternal and fetal tissues in sterile 0.1 M PBS pH 7.4 under a stereomicroscope. For culturing, the tissue was fragmented in small pieces and incubated in collagenase IA solution (3.6 mg/mL, Sigma C6885) and protease (dispase, 1.2 mg/mL, Sigma P3417) for 30 min, at 37 °C to dissociate the cells, this being stopped by adding Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., St Louis, MO, EUA) with 20% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA). The samples were centrifuged at 1000 rpm, for 5 min at 4 °C and the pellet resuspended in 1 mL DMEM supplemented with 10% FBS, and antibiotics (penicillin/streptomycin, 0.5 mg/mL, Vitrocell-Embryolife, Campinas, SP, Brazil). Cell number and viability rates were assessed by Trypan Blue dye exclusion (Sigma). Cells were plated at $2.5 \times 10^5$ cells/well on glass coverslips or directly on 24-well culture plates, and others at $1 \times 10^4$ cells/well on 96-well plates (TPP Techno Plastics Products AG, Switzerland). The cells were maintained in DMEM supplemented with 0.5 mg/mL calcium lactate, 0.2 μg/mL insulin, 4 mg/mL BSA (Albumin from Bovine Serum, Sigma), 1 μg/mL Mito plus Serum Extender (BD Biosciences, Franklin Lakes, NJ, USA), 0.1 mM L-glutamine (Sigma), antibiotics (penicillin/streptomycin 0.5 mg/mL) and 10% FBS in a