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► **To cite this version:**

Natalia Tkachuk, Julia Kiyan, Sergey Tkachuk, Roman Kiyan, Nelli Shushakova, et al.. Urokinase induces survival or pro-apoptotic signal in human mesangial cells depending on the apoptotic stimulus. *Biochemical Journal*, 2008, 415 (2), pp.265-273. 10.1042/BJ20071652 . hal-00478936

**HAL Id: hal-00478936**

**<https://hal.science/hal-00478936>**

Submitted on 30 Apr 2010

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MS Tkachuk N, RE: BJ2007/1652

## Urokinase induces survival or pro-apoptotic signal in human mesangial cells depending on the apoptotic stimulus

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Short title: uPA/uPAR in mesangial cells apoptosis

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Abbreviations used: ATF, amino-terminal fragment of uPA; BAD, Bcl2/Bclx-L-antagonist of cell death; FCS, fetal calf serum; GPI, glycosylphosphatidylinositol; HG, medium containing 30mM D-glucose; MCs, human mesangial cells; MEK/ERK, mitogen-activated protein kinase/extracellular regulated kinase; MTR, MitoTracker Red; M6P/IGF2R, mannose-6-phosphate/insulin growth factor 2 receptor; NC, negative control for apoptosis; PI3-K/AKT, phosphatidylinositol-3-kinase/protein kinase B; p38-MAPK, p38-mitogen-activated protein kinase; RGD, Arg-Gly-Asp; siRNA, small interfering RNA; SF, serum-free medium; TU, transducing units; TUNEL, Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling; uPA, urokinase; uPAR, urokinase receptor.

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## SYNOPSIS

Deregulated apoptosis of mesangial cells (MCs) is associated with a number of kidney diseases including end-stage diabetic nephropathy. Cell death by apoptosis is a tightly orchestrated event, whose mechanisms are not completely defined. We show that the urokinase (uPA)/urokinase receptor (uPAR) system can initiate both cell survival and pro-apoptotic signals in human MCs in response to different apoptotic stimuli. uPA abrogated MCs apoptosis induced by serum withdrawal conditions and enhanced apoptosis initiated in MCs by high glucose. Effects of uPA were independent of its proteolytic activity and required uPAR for both pro- and anti-apoptotic effects of uPA. Studies on uPAR interactome provide evidence that the opposing effects of uPA were directed via different uPAR-interacting transmembrane partners. Exposure of MCs to RGD peptide led to abrogation of anti-apoptotic effect of uPA that implies involvement of integrins in this process. A pro-apoptotic effect of uPA under high glucose conditions was mediated via association of uPAR and the cation-independent mannose-6-phosphate (M6P)/insulin growth factor 2 receptor (IGF2R). Both receptors were coprecipitated and colocalized in MCs. Studies on the underlying signaling indicate that the ERK1/2, AKT and BAD protein were involved in regulation of apoptosis by uPA in MCs. M6P/IGF2R mediated BAD perinuclear localization during apoptosis initiated by uPA and high glucose. In conclusion, we provide evidence that in MCs the uPA/uPAR system regulates survival/apoptosis processes in a stimulus-specific fashion via mitochondria-dependent mechanism and that BAD protein serves as a downstream molecule.

**Keywords:** cell signalling, cell survival, cell death, urokinase receptor, mannose-6-phosphate (M6P)/insulin growth factor 2 receptor (IGF2R), BAD protein.

## INTRODUCTION

One of the major consequences of diabetes is the end-stage renal failure associated with expansion of mesangial extracellular matrix and increased proliferation of mesangial cells (MCs) [1]. Apoptosis of MCs is believed to play a critical role in regulation of these processes [2]. The underlying molecular mechanisms, although being intensively investigated, still remain uncertain. Recent findings coming from *in vitro*, *in vivo* and clinical studies point to the important role of the fibrinolytic urokinase (uPA)/urokinase receptor (uPAR) system in kidney diseases including diabetes-related end-stage renal failure [3, 4]. uPAR is supposed to be involved in the prevention of renal fibrosis in obstructive nephropathy [5]. Upregulation of uPA and uPAR were shown in crescentic glomerulonephritis and in a murine model of nephrotoxic nephritis correspondingly [6, 7]. Diabetic rats exhibited high levels of expression of uPA and uPAR, mainly in MCs, that was associated with mesangial expansion [8]. However, there is no data on the involvement of the uPA/uPAR system in regulation of MCs apoptosis and underlying mechanisms are not yet clarified.

A body of evidence shows that the uPA/uPAR system can protect cells from undergoing apoptotic cell death [9]. Nontransformed human retinal pigment epithelial cells pre-exposed to uPA exhibited reduced susceptibility to anoikis and UV-induced apoptosis through the activation of the MEK/ERK and PI3-K/AKT survival pathways [10]. Simultaneous down-regulation of uPAR and uPA in SNB19 human glioma cells activated caspase-mediated apoptosis [11]. When uPAR was inhibited in different metastatic cell lines, the expression of members of the Bcl-2 family (Bax, Bcl-2, Bak and Bcl-xL) was changed in a pro-apoptotic manner [12]. On the other hand, several recent findings documented a pro-apoptotic role of uPA and uPAR in cellular physiology. Thus, a negative effect of uPA on cell survival has been observed in a mouse model of retinal damage [13]. Cleaved high molecular weight kininogen via binding to uPAR induced apoptosis of human endothelial cells, most likely due to changes in uPAR association with other signaling components [14]. Together, these data suggest multifunctional properties of the uPA/uPAR system in survival/apoptosis processes. Since uPAR is linked to the outer membrane leaflet by a GPI-anchor, all of its diverse biological functions are strictly dependent on its interactions with other proteins. However, the important question, whether cell survival or apoptotic response to uPA may be determined by the composition of the uPAR signaling receptor complex has not been addressed so far.

In the present study, we investigated effect of uPA on apoptosis of human MCs under high glucose and growth factor withdrawal conditions. We demonstrate that uPA can initiate both cell survival and apoptotic signals depending on the apoptotic stimulus. Our results indicate that the pro-apoptotic effect of uPA requires interaction of uPAR with the cation-independent mannose-6-phosphate (M6P)/insulin growth factor 2 receptor (IGF2R), whereas the anti-apoptotic action of uPA is mediated by integrins. Thus, the uPA/uPAR signals are transduced through two mechanisms with apoptotic stimulus acting as the switch.

## EXPERIMENTAL

### Cell culture

Primary human mesangial cells were obtained from Lonza. The cells were cultured in a medium recommended by the supplier and were used in passages 6 and 7. Cells were serum-starved for 24 hours followed by incubation with 10 nM uPA (or with 10 nM amino-terminal fragment of uPA (ATF), where indicated) in combination with serum-free medium (SF) or with high glucose medium containing 30 mM D-glucose (HG) for indicated times. In some experiments cells were pre-treated with 10  $\mu$ M RGD (Arg-Gly-Asp) peptide from Bachem or with 4mM D-mannose 6-phosphate sodium salt (M6P) obtained from Sigma or with 0.4  $\mu$ g/ml neutralizing polyclonal anti-uPAR antibody from R&D Systems or with 1 mM EDTA (Roth). Human uPA (predominately of the high molecular weight form, 100 000 IU, 1mg/ml) was



purchased from Loxo GmbH, ATF of human uPA (1mg/ml) was from Innovative Research. For apoptosis detection the cells were stained with Hoechst 33258 (Sigma).

### **Apoptosis detection**

#### **Hoechst 33258 staining assay**

MCs were seeded onto glass coverslips in 12-well plastic plate (Nunc™) and cultured in standard culture medium. After 24 h starvation the cells were treated as described in above for 24-72 h. Cells in culture medium were used as a negative control for apoptosis (NC). D-Mannitol was used as an osmotic control. The cells were fixed with 4% (w/v) paraformaldehyde in PBS pH=7.4 for 10 min, washed with PBS, stained with 2µg/ml Hoechst 33258 for 30 min at 37°C and embedded in Vectashield mounting media (Vector Laboratories). The cells were observed under Leica DM LB fluorescence microscope (Leica Microsystems), equipped with 40x objective by the observer blind to the cell treatment. The nuclei stained with Hoechst 33258 that were markedly bright and small or divided into several homologous chromatin clumps, were defined as being condensed or fragmented nuclei, respectively. Cells from ten random microscopic fields of view at 400x magnification were counted per coverslip and the average % of apoptotic cells was calculated.

#### **TUNEL assay**

MCs apoptosis was examined with commercially available DeadEnd™ Fluorometric TUNEL System (Promega) following the manufacturer's instruction. Subsequently, fluorescence microscopy (Leica Microsystems) analyses were performed. Cells from ten random microscopic fields of view were counted per coverslip.

#### **Caspase 3/7 activity assay**

Caspase-3/7 activity was determined using a Caspase-Glo™3/7 assay (Promega) according to the manufacturer's protocol.

#### **Preparation of mitochondrial fraction**

Pure mitochondrial preparation in case of uninfected and crude mitochondrial preparations in case of infected with lentivirus MCs were obtained exactly as described previously [15, 16].

#### **Immunoprecipitation and Western blotting**

For immunoprecipitation subconfluent serum-starved MCs were treated with stimuli as described in above for 50 min at 37°C, lysed and precleared. Precleared cell lysates containing 1.3-1.7 mg of total protein were incubated for 2 h with appropriate antibodies and then overnight at 4°C with protein A/G PLUS-agarose. Precipitates were washed in PBS buffer containing inhibitors cocktail and subjected to SDS-PAGE.

For Western blotting the proteins were transferred to PVDF membrane (Roche Diagnostics). Membranes were probed with appropriate antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). The immune complexes were visualized by an enhanced chemiluminescence detection system (Perkin Elmer Life Sciences). Chemiluminescent images were captured using VersaDoc-3000 (Bio-Rad Laboratories) and quantified using Quantity One software (Bio-Rad Laboratories).

The following primary monoclonal antibodies were used: anti-uPAR clone R3 (Monozyme), anti-uPAR antibody recognizing domains D2+D3 (American Diagnostica), anti-diphospho-ERK1/2 (Sigma), anti-ERK and anti-BAD (Transduction Laboratories), anti-β-tubulin (BD Bioscience Pharmingen), anti-ATP synthase β-chain (Molecular Probes), anti-Bcl-2 antibody (DakoCytomation). The primary polyclonal antibodies were: anti-M6P/IGF2R (AF2447) (R&D Systems), anti-M6P/IGF2R (H-300) (Santa Cruz Biotechnology), anti-

phospho-AKT (Ser473) (193H12) and anti-AKT (Cell Signaling Technology), anti-Bcl-x<sub>s/L</sub> (Santa Cruz Biotechnology). The following secondary antibodies were used: Alexa Fluor 488-conjugated donkey anti-goat and Alexa Fluor 594-conjugated goat anti-mouse antibodies (Molecular Probes), peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). Normal rabbit IgG and normal mouse IgG were from Upstate Biotechnology.

### **Lentiviral vector production and cell infection**

Lentiviral siRNA vectors were designed and cloned as described previously [17]. Lentiviral vectors were produced by transient transfection of 293T cells using an established procedure [18]. MCs were infected in the presence of 8 µg/ml polybrene with viruses at 10<sup>8</sup> TU/ml and used for experiments day 3 after infection. 95% of cells have been shown to be infected under these conditions.

### **Cell transfection**

Human primary MCs were transfected using Nucleofector™ (Amaxa Biosystems). Basic Primary Smooth Muscle cell nucleofector kit (Amaxa Biosystems) was used according to the manufacturer's instructions. Briefly, the cells were resuspended in the nucleofector solution. 100 µl of cell suspension at a density of 5×10<sup>5</sup>-1×10<sup>6</sup>/ml mixed with 5 µl of 10 µM siRNA were transferred to a cuvette and nucleofected with A-33 program an Amaxa nucleofector apparatus. The cells were immediately transferred into wells of 12-well plate containing 37° C pre-warmed culture medium. Cells treated with nonspecific RNA were used as a control. MCs were used for experiments day 3 after nucleofection.

### **Surface-labelling of receptors and immunofluorescence confocal microscopy**

Cells were seeded and cultured on glass coverslips. Serum-starved MCs were treated for 15 min with 10 nM uPA in combination with HG or with SF. After stimulation, cells were washed with PBS and incubated with anti-uPAR monoclonal antibodies and anti-M6P/IGF2R polyclonal antibody in 0.1% (w/v) sodium azide with 1% (w/v) BSA solution to prevent internalization at 4°C for 15 min. Cells were washed with ice-cold PBS and incubated with Alexa 594-conjugated and Alexa 488-conjugated secondary antibodies respectively in sodium azide solution at 4°C for 15 min and fixed in methanol -20°C for 2 min. After fixation, cells were embedded in Poly-Mount mounting media (Polysciences). The fluorescence cell images were captured using a Leica TCS-SP2 AOBs confocal microscope (Leica Microsystems). The images were taken with oil-immersed 63x objective, NA=1.4 under the same microscope tuning. For evaluation analysis, the immunocytochemical images were transformed to grayscale colour mode and the staining intensity of cells was quantitated using Quantity One software (Bio-Rad Laboratories). For each sample, six images from three independent experiments were analysed.

### **Enzyme-linked immunosorbent assay**

Enzyme-linked immunosorbent assay (ELISA) kits for human uPA and PAI-1 were purchased from American Diagnostica Inc. and R&D Systems respectively. uPA and PAI-1 contents in conditioned media of MCs treated as described in above were measured for 24, 48, 72 hours according to the manufacturer's instructions.

### **Statistical analysis**

Data are presented as mean value ± SEM. Statistical analysis was performed using Student *t*-test. *P*<0.05 was considered significant.

## RESULTS

### uPA elicits anti- and pro-apoptotic effects in MCs under different apoptotic conditions

To investigate a role of the uPA/uPAR system in apoptosis of MCs, we first examined whether addition of uPA could influence apoptosis induced by two different stimuli: serum-free medium and high glucose medium containing 30 mM D-glucose. The results of Hoechst 33258 staining show that the both stimuli induce apoptosis in MCs within 24-72 h of cell treatment in a time-dependent manner. The addition of uPA abrogated apoptosis induced in MCs by SF medium. By contrast, uPA in combination with HG resulted in a 1.8-fold increase of apoptosis in comparison with that induced by HG alone (Figure 1A). These opposing effects of uPA were confirmed by TUNEL staining (Figure 1B) and by measurement of caspase 3/7 activity (Figure 1C). These results indicate that uPA can elicit both pro-apoptotic and anti-apoptotic effects on MCs under different conditions.

### ERK1/2, AKT and BAD are downstream molecules mediating effects of uPA

The results presented above suggest that uPA might activate different signaling pathways under different apoptotic conditions. Therefore, we investigated effect of uPA on phosphorylation of ERK1/2 and AKT, the key pathways in regulation of a balance between apoptosis and survival [19]. Since rapid changes in phosphorylation of these kinases preceding downstream apoptotic events have been reported [20, 21], we examined ERK1/2 and AKT after 15-20 min and 3 h of MCs stimulation. A 2.1-fold increase in phosphorylation of ERK1/2 was observed after 15 min uPA stimulation in SF; this effect remained significant, although less pronounced after 3 h. By contrast, when we added uPA to HG, an inhibition of ERK1/2 phosphorylation was detected, which was more pronounced after 3 h cell stimulation (Figure 2A). Similar effects were observed for AKT phosphorylation (Figure 2B). High phosphorylation levels of ERK1/2 and AKT in normal culture medium containing 5% (v/v) FCS used as a negative control for apoptosis was detected (data not shown). We observed no changes in the regulation of the p38-MAPK previously reported to mediate apoptosis in other cell types [22]. Taken together, these data indicate that the ERK1/2 and AKT, but not p38 pathway might mediate regulation of apoptosis by uPA in MCs.

We next asked which proteins might be involved as downstream mediators of the effects of uPA on apoptosis in MCs. We addressed the Bcl-2 family of proteins, which plays a key role in apoptotic event [23]. We did not find any difference in protein level of Bcl-2 and Bcl-x<sub>S/L</sub> – the anti-apoptotic members of that group (data not shown) and finally analysed pro-apoptotic BAD protein. Although no changes in short or long-term BAD phosphorylation were found in these experiments, we observed an increase of BAD protein in mitochondrial fraction of MCs under uPA/HG conditions after 48-72 h cell stimulation (Figure 2C). In response to uPA/SF, BAD content was decreased thus supporting our data on the anti-apoptotic function of uPA under these conditions. These results suggest a requirement for BAD protein downstream of ERK and AKT in the induction of the uPA-mediated effects on MCs apoptosis/survival.

### uPAR is involved in both cell survival and apoptotic effects of uPA

To determine the role of uPAR in uPA-directed apoptosis of MCs, cells with down-regulated uPAR expression (LV-siuPAR MCs) were used (Figure 3A). Activation of both the ERK1/2 and AKT was strictly dependent on uPAR, since down-regulation of uPAR prevented phosphorylation of both kinases. Consequently, siuPAR cells demonstrated negligible changes in amount of BAD in cell mitochondrial fractions in response to uPA under different apoptotic conditions. These data assign a direct role to uPAR in controlling both pro- and anti-apoptotic effects of uPA. The increased basal level of BAD protein was observed in MCs with down-regulated uPAR that can illustrate an important regulating role for uPAR in apoptotic process.

### **Effects of uPA are independent of its proteolytic activity**

To exclude any effect of proteolytic activity of uPA on apoptosis of MCs, amino-terminal fragment of uPA devoid of any catalytic activity but still binding to uPAR with a high affinity, was used for cell treatment. The results of Hoechst 33258 staining indicate that effects of ATF on apoptosis of MCs were identical to those of uPA (Figure 3B). Thus, addition of ATF to HG resulted in a 1.6-fold increase of apoptosis as compared to apoptosis induced by HG alone. By contrast, ATF decreased in a 2-fold apoptosis initiated by growth factor withdrawal. To exclude a possible influence of changes in endogenous uPA and PAI-1 in MCs on the observed effects, extracellular concentration of uPA and PAI-1 was determined by means of specific ELISA for different time points (24, 48, 72 hours). Although a moderate time-dependent increase in PAI-1 and uPA was observed within 72 hours, no changes in uPA and PAI-1 concentration were found in response to HG and serum deprivation.

### **Anti-apoptotic effect of uPA is mediated by integrins**

In various cell types uPAR has been shown to associate functionally with different integrins [24]. Integrin-mediated adhesion has been reported to support cell survival by signaling downstream of ERK1/2 [25]. To check whether integrins might be involved in the uPA/uPAR-directed anti-apoptotic effect in MCs, cells were pre-treated with arginine-glycine-aspartate (RGD) peptide that could mimic cell adhesion proteins and bind to integrins. This treatment had a negligible effect on the HG and uPA/HG induced apoptosis, as shown by Hoechst 33258 staining (Figure 4). SF medium-induced apoptosis was independent of integrins. At the same time, RGD completely abrogated the pro-survival effect of uPA on the SF medium-induced apoptosis.

### **Pro-apoptotic but not survival effect of uPA requires uPAR interaction with M6P/IGF2R**

Reports on the involvement of M6P/IGF2R in apoptotic events and its ability to interact with uPAR [26] prompted us to suggest that the binding of uPAR to M6P/IGF2R might be involved in the pro-apoptotic effect of uPA in MCs. We performed coimmunoprecipitation using anti-M6P/IGF2R antibody from MCs stimulated with uPA under different apoptotic conditions. In all immunoprecipitates we observed a basal signal for uPAR that could indicate constitutively presented uPAR-M6P/IGF2R complex in MCs. However, the interaction between uPAR and M6P/IGF2R in uPA/HG treated MCs was significantly enhanced in comparison with cells treated with HG only. uPAR-M6P/IGF2R association under SF conditions without and with uPA remained at the basal level (Figure 5A). To check the specificity of this interaction for the uPA/HG initiated cellular events, several experimental settings have been performed (Figure 5A). MCs were pre-treated with M6P, a natural ligand for M6P/IGF2R. This treatment completely abrogated formation of uPAR-M6P/IGF2R complex. To verify a role of uPAR in this association, cells were pre-treated with uPAR blocking antibody. The uPA-induced association of M6P/IGF2R – uPAR was abrogated in this case.

These data together with the observation that RGD peptide blocked the uPA-induced cell survival effect supposed that anti- and pro-apoptotic functions of uPA under different conditions might be mediated via uPAR association with two different transmembrane signaling proteins, namely integrins or M6P/IGF2R. Therefore, to test, whether uPAR association with integrins was specific for uPA anti-apoptotic action, integrins targeting was finally performed using cell treatment with EDTA. As shown in Figure 5A, this treatment did not affect uPAR-M6P/IGF2R association under HG and uPA/HG conditions. At the same time, uPAR-M6P/IGF2R association in response to uPA under SF conditions, which was otherwise non-significant, was increased after EDTA treatment. These data supports results of our experiments with RGD peptide and show that the anti-apoptotic effect of uPA initiates



uPAR-integrins association. They further imply that if integrins are blocked, a corresponding “pool” of uPAR remains unbound and might therefore interact with other adaptor proteins on the cell surface including M6P/IGF2R. Together, these results confirm our conclusion that pro- and anti-apoptotic effects of uPA utilize uPAR association with different interacting partners.

In a parallel set of experiments shown in Figure 5B, we further investigated association between uPAR and M6P/IGF2R using immunocytochemistry. The amount of uPAR on the cell surface was invariant under different conditions used and the amount of M6P/IGF2R on the cell surface on MCs incubated in SF medium without and with uPA was marginal. HG upregulated M6P/IGF2R on the cell surface and initiated partial colocalization of M6P/IGF2R and uPAR, which was further increased in response to uPA. Interestingly, cell surface presentation of M6P/IGF2R was dependent on uPAR, since down-regulation of uPAR in MCs by means of siRNA resulted in M6P/IGF2R increase. Moreover, addition of different apoptotic stimuli did not affect the M6P/IGF2R receptor in these cells (Figure 5B, lower row). These data confirm the requirement of uPAR - M6P/IGF2R interaction for the pro-apoptotic effect of uPA and suggest an important role of uPAR in M6P/IGF2R regulation.

### **M6P/IGF2R regulates BAD translocation to nuclear membranes during apoptosis**

Recent reports emphasize the importance of intracellular redistribution of proteins associated with apoptotic events. In particular, their perinuclear localization as a requirement for final apoptotic steps has been suggested [27]. Since we found that BAD protein serves as a downstream effector molecule in the uPA/uPAR/M6P/IGF2R-initiated apoptotic events in MCs, we next examined changes in BAD intracellular locations under different experimental conditions (Figure 6). Treatment of control cells with SF medium resulted in the formation of perinuclear aggregations of BAD protein together with mitochondria. Addition of HG aggravated the BAD protein clustering around the nuclei. Redistribution of BAD was mostly pronounced in cells treated with uPA/HG combination. By contrast, MCs treated with uPA/SF medium revealed no BAD protein translocation. When M6P/IGF2R was down-regulated in MCs (siM6P/IGF2R), no change in the distribution of BAD in response to uPA/HG was observed. These results rule out a primary role for M6P/IGF2R in propagation of a pro-apoptotic signal in MCs. They further suggest that the association of uPAR with pro-apoptotic adaptor proteins on the cell surface might lead to deep change in mitochondria, their intracellular redistribution and perinuclear accumulation together with related proteins and finally to cell death.

### **DISCUSSION**

Despite a central role of MCs in regulation of glomeruli associated kidney diseases [28], remarkably little is known of the mechanisms, by which survival and apoptosis of MCs are mediated. In the current study, we provide convincing evidence that uPA via its specific receptor uPAR and the association of uPAR with adaptor proteins on the cell surface regulates survival/apoptosis processes in human MCs. Using two different apoptotic stimuli – SF medium and HG – we show that the association of uPAR with cell surface proteins is stimulus-specific and results in anti-apoptotic effect of uPA under growth factors withdrawal conditions and in pro-apoptotic effect of uPA under high glucose conditions. We demonstrate that the anti-apoptotic effect of uPA is mediated by the RGD-dependent integrins, whereas its pro-apoptotic effect is conditioned by interaction of uPAR with M6P/IGF2R. We show that uPA acts through the MEK/ERK- and PI3-K/AKT signaling pathways. We report that the uPA/uPAR system regulates survival/apoptosis processes via mitochondria-dependent mechanism and that BAD protein serves as a downstream molecule. To our knowledge, this is the first study to document a functional link between uPAR - M6P/IGF2R and BAD protein.

Numerous reports documented that uPA-uPAR interaction activates ERK and PI3-K pathways [29, 30]. The role of integrins in regulation of cell survival has been well established [31]. In our experiments, uPA induced strong activation of ERK and AKT under SF conditions resulting in the anti-apoptotic effect of uPA on MCs. Blocking of integrins with RGD peptide converted the anti-apoptotic effect of uPA to a pro-apoptotic, suggesting an important role for the RGD-dependent integrins in this response. We suggest that two mechanisms might be involved. Thus, uPA under SF conditions could promote interaction of uPAR with RGD-dependent integrins similar to the reported activation of uPAR and  $\beta 1$  integrin interaction in response to uPA [32]. Alternatively, uPA itself could engage through its individual domains two different surface receptors, namely uPAR and integrin, as reported earlier [33]. Under HG conditions, uPA fails to activate ERK and AKT pathways suggesting a disruption of the uPAR-integrins interaction. Integrins targeting by RGD peptide and EDTA confirmed a minor role of integrin-uPAR functional interference for the pro-apoptotic events and rather suggest an important role for M6P/IGF2R under those conditions.

The M6P/IGF2R has been implicated as a growth and tumor suppressor and receptor for rapid induction of apoptosis [26]. In the insulin-secreting cell lines and in the human erythroleukemia cell line HG enhances presentation of M6P/IGF2R at the cell plasma membrane [34]. Our immunocytochemical studies demonstrate that MCs under SF conditions have a negligible amount of M6P/IGF2R on the cell surface, whereas HG up-regulates this receptor. We propose that under HG conditions, uPA induces cooperation between uPAR and M6P/IGF2R resulting in their colocalization on the cell surface and perhaps in delaying their internalization. Mechanism of M6P/IGF2R redistribution to plasma membrane associated with decreased internalization was already reported [35]. Our experiments using ATF speak in favour of decreased internalization of uPAR and M6P/IGF2R in MCs under uPA/HG conditions. ATF, which serves as the binding domain for uPA, can not be internalized, since the catalytic domain of uPA is needed for internalization. In our experiments, ATF, similarly to uPA, induced colocalization of uPAR and M6P/IGF2R on the cell surface under ATF/HG conditions (data not shown). It should be pointed out that ATF completely retained both pro- and anti-apoptotic effects of uPA under the conditions used. The results of experiments with ATF presented here emphasize the role of uPA binding to uPAR and consequently the role of uPAR itself in survival/apoptosis processes.

In our experiments on MCs with silenced uPAR, we observed abrogation of the uPA effects. One possible explanation for this observation is that down-regulation of uPAR diminished association of uPAR with other interacting molecules on the cell surface and the signaling complexes following uPA-uPAR binding were turned off. Interestingly, we noticed that silencing of uPAR markedly upregulated the basal level of BAD protein and M6P/IGF2R in MCs suggesting that uPAR itself is an anti-apoptotic factor. uPAR activation by uPA followed by signaling complex formation in a stimulus-specific fashion, might enable uPAR to operate as a pro-apoptotic factor.

Our study demonstrates that BAD is a key signaling molecule, which mediates both pro-apoptotic and anti-apoptotic processes in human MCs in response to uPA. Our results indicate that in MCs, BAD protein acts via at least two different mechanisms: the change of its amount in mitochondria and intracellular redistribution. Under SF conditions, uPA prevented apoptosis by decrease of BAD amount in mitochondrial fraction and no redistribution of BAD was observed. By contrast, under HG conditions uPA induced apoptosis by increasing of BAD amount in mitochondrial fraction and BAD was found in a perinuclear region. These findings confirm the recent reports of others, which suggest that in response to the apoptotic stimulus, subpopulations of mitochondria and some regulatory proteins are mobilized to translocate to the highly metabolically active perinuclear ring, to affect the process of apoptosis [36].



Overall, our results support a model in which the uPA/uPAR system via association with other receptors on the cell surface regulates survival/apoptosis processes in MCs in a stimulus-dependent manner. These findings may help to unravel an unexplored link between the uPA/uPAR system and regulation of cell fate upon diabetic nephropathy and may serve as a starting point to the understanding of molecular mechanisms underlying kidney pathology.

### ACKNOWLEDGMENTS

We are grateful to Frank Hausadel for excellent technical assistance. This work was supported by grant SH 98/1-1 from the Deutsche Forschungsgemeinschaft.

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## FIGURE LEGENDS

**Figure 1. Pro-apoptotic or anti-apoptotic effects of uPA on MCs depend on apoptosis-inducing stimuli.** Apoptosis was induced in quiescent MCs by incubation in serum-free medium (SF) or in high glucose medium containing 30 mM D-glucose (HG) in the absence or presence of 10 nM uPA. MCs incubated in culture medium containing 5% (v/v) FCS were used as a negative control (NC) for apoptosis. **(A)**, The apoptotic response was investigated by Hoechst 33258 staining of MCs incubated for 24-72 h as described in above. Data are expressed as a percentage of apoptotic cells and presented as the mean  $\pm$  SEM (n=10). \*  $P < 0.05$ , \*\* $P < 0.01$  versus negative control (NC), # $P < 0.05$ , ## $P < 0.01$  versus treatment without uPA. **(B)**, TUNEL staining of MCs was performed after cells incubation for 24-72 h as described in above and quantified by fluorescence microscopy. Data are expressed as a percentage of increased amount of apoptotic cells and presented as the mean  $\pm$  SEM (n=10). \*  $P < 0.05$ , \*\* $P < 0.01$  versus negative control (NC), # $P < 0.05$ , ## $P < 0.01$  versus treatment without uPA. **(C)**, Caspase-3/7 activation was measured in MCs stimulated for 24-72 h as indicated in above by luminescent assay. Data are expressed in arbitrary units and presented as the mean  $\pm$  SEM (n=5). \* $P < 0.05$ , \*\* $P < 0.01$  versus negative control (NC), # $P < 0.05$ , ## $P < 0.01$  versus treatment without uPA.

**Figure 2. ERK1/2, AKT and BAD protein are downstream molecules mediating the effects of uPA in MCs under different apoptotic conditions.** Quiescent MCs were treated with 10 nM uPA in combination with HG or with SF medium for 15-20 min and for 3 h; whole cell lysates were immunoblotted with antibody against phosphorylated ERK1/2 protein **(A)** and phosphorylated AKT **(B)**, followed by membrane stripping and immunoblotting with appropriate antibody to detect a gel loading (shown on the lower panel). Folds shown are the representative result of three independent experiments. **(C)**, BAD protein was visualized by immunoblotting with specific antibody in pure mitochondrial fraction of MCs incubated with indicated stimuli for 48 h and 72 h. The lower panel presents a loading control. Folds shown are the representative result of three independent experiments.

**Figure 3. uPAR is involved in both pro-apoptotic and anti-apoptotic effects of uPA.** **(A)**, MCs were infected with LV-siuPAR or empty (LV-mock) viruses followed by stimulation with 10 nM uPA for indicated times; whole cell lysates were immunoblotted with antibody against phosphorylated ERK1/2 and phosphorylated AKT; BAD protein in crude mitochondrial fraction was visualized by immunoblotting with specific antibody. Folds shown are the representative result of three independent experiments. Anti-ERK1/2, anti-AKT and anti-ATP synthase antibodies were used for a loading control. **(B)**, Quiescent MCs were incubated for 48 h with 10 nM uPA or 10 nM ATF in combination with HG or with SF medium. Cells were then fixed and stained with Hoechst 33258. Nuclei were visualized by fluorescence microscopy. Data are expressed as a percentage of apoptotic cells and presented as the mean  $\pm$  SEM (n=5). \* $P < 0.05$  versus SF medium, # $P < 0.05$  versus HG treatment.

**Figure 4. Anti-apoptotic effect of uPA is mediated by integrins.** Quiescent MCs were treated with 10  $\mu$ M RGD peptide for 1 h followed by incubation with 10 nM uPA in combination with HG or with SF medium for 48 h. Cells were then fixed and stained with Hoechst 33258. Nuclei were visualized by fluorescence microscopy. Data are expressed as a percentage of apoptotic cells and presented as the mean  $\pm$  SEM (n=5). \* $P < 0.05$  versus SF medium, # $P < 0.05$  versus HG treatment.

**Figure 5. Pro-apoptotic but not survival effect of uPA requires uPAR interaction with M6P/IGF2R.** (A), Quiescent MCs were left untreated or were pre-treated at 37°C with 4 mM D-mannose 6-phosphate sodium salt (M6P) for 1 h or with 0.4 µg/ml polyclonal anti-uPAR antibody for 1 h or with 1 mM EDTA for 30 min followed by incubation with 10 nM uPA in combination with HG or with SF medium for 50 min at 37°C. Anti-M6P/IGF2R antibody was used to coimmunoprecipitate uPAR from cell lysates. The immunoprecipitates were analyzed with anti-uPAR antibody (upper panel). The lower panel represents statistic data of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  versus negative control (NC), # $P < 0.05$ , ## $P < 0.01$  versus treatment without uPA. (B), Double-immunofluorescence surface-labelling for uPAR (1<sup>st</sup> row), for M6P/IGF2R (2<sup>nd</sup> row) and merge image (3<sup>rd</sup> row) in MCs after stimulation with 10 nM uPA in combination with HG or with SF medium for 15 min. Immunofluorescence surface-labelling for M6P/IGF2R in MCs with down-regulated uPAR is presented in the 4<sup>th</sup> row. Cells in 5% (v/v) FCS containing culture medium were used as a negative control (NC). Shown are the representative images of three independent experiments.

**Figure 6. Association of uPAR with M6P/IGF2R induces subcellular redistribution of BAD protein in MCs.** MCs were nucleofected with non-specific RNA (upper three rows) or siM6P/IGF2R RNA duplexes (lower three rows) followed by incubation with 10 nM uPA in combination with HG or with SF medium for 48 h; BAD protein (green) was visualized by immunostaining with anti-BAD antibody and Alexa 488-conjugated secondary antibodies, mitochondria (red) were visualized by staining with MitoTracker Red (MTR). Merge image is presented in yellow. Cells in 5% (v/v) FCS containing culture medium were used as a negative control (NC) for apoptosis. Shown are the representative images of three independent experiments.

Figure 1

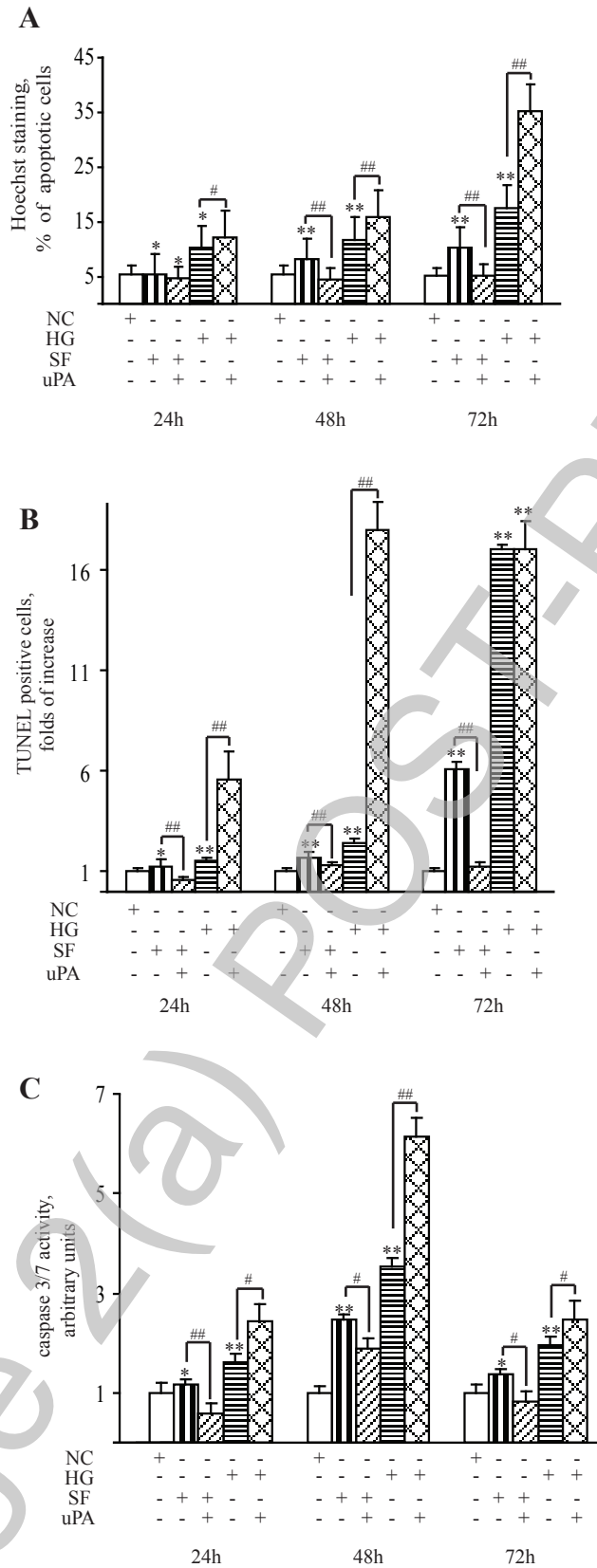




Figure 2

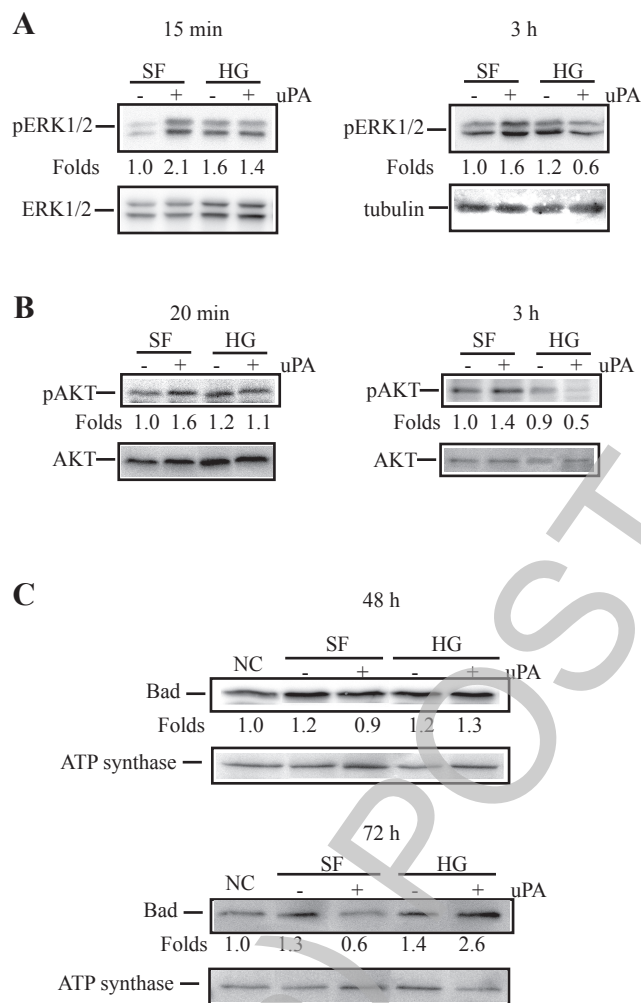
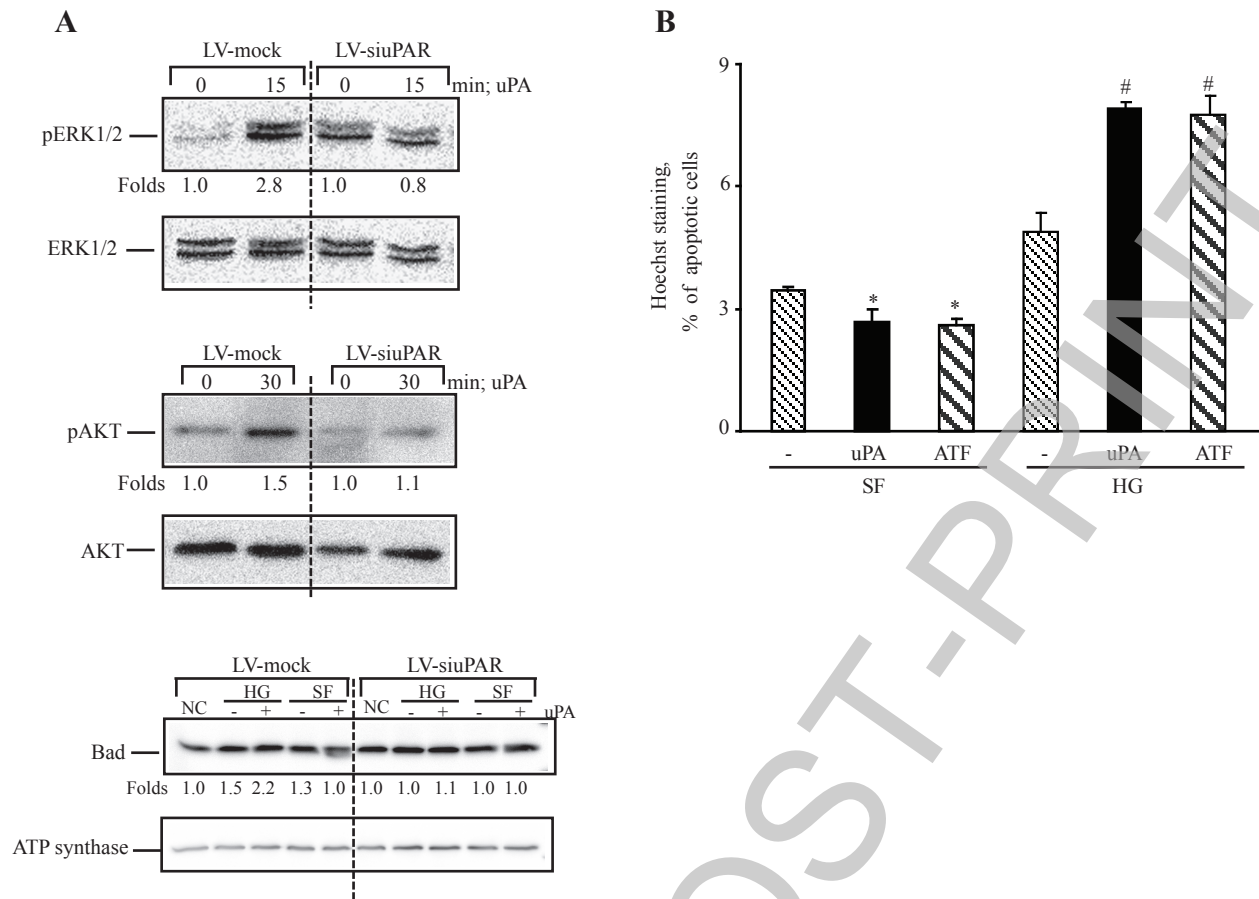
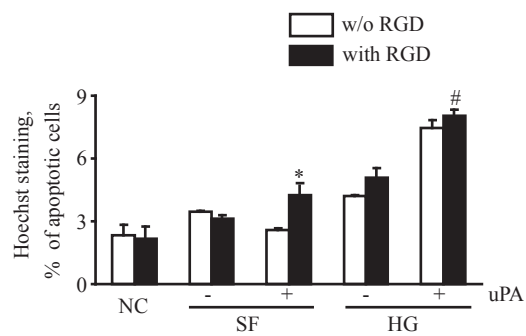


Figure 3



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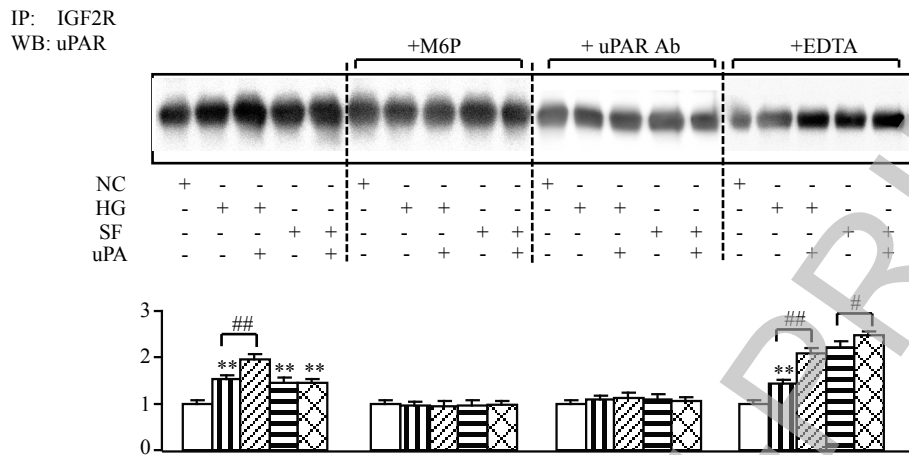
Figure 4



Stage 2(a) POST-PRINT

Figure 5

A



B

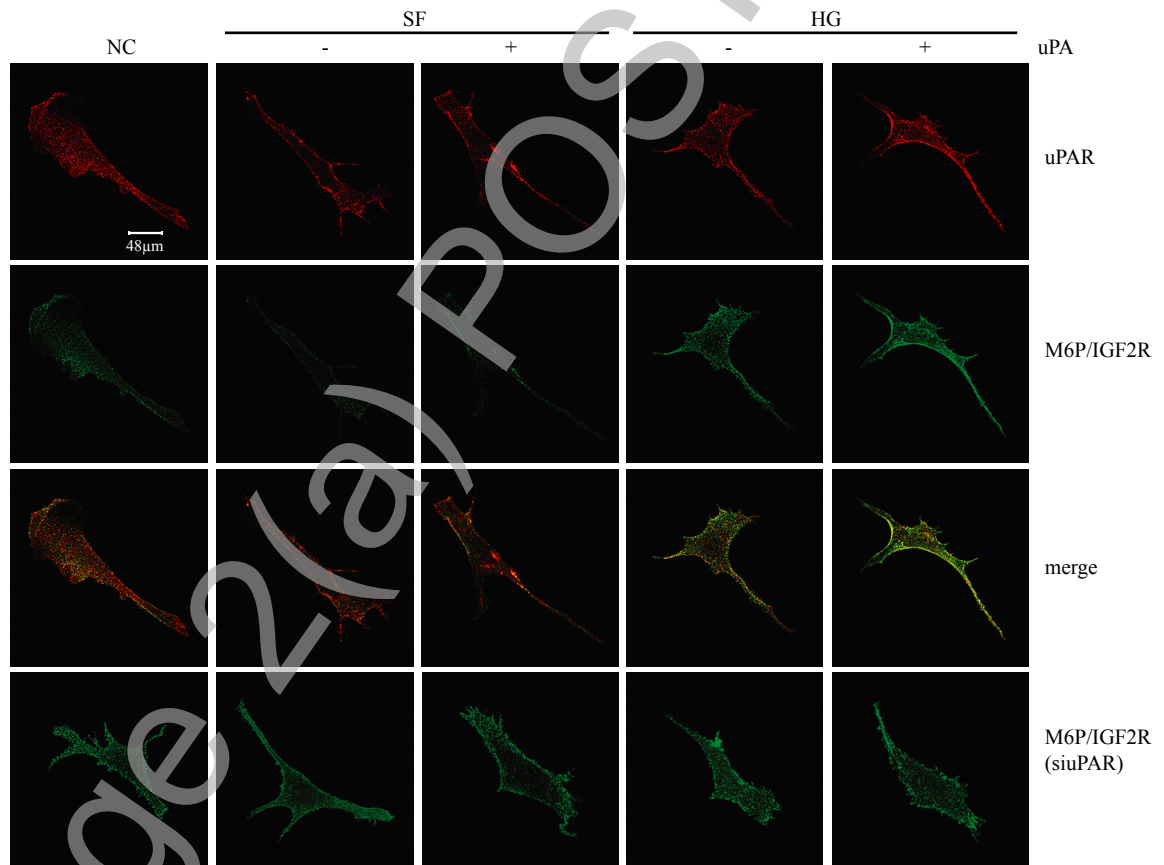
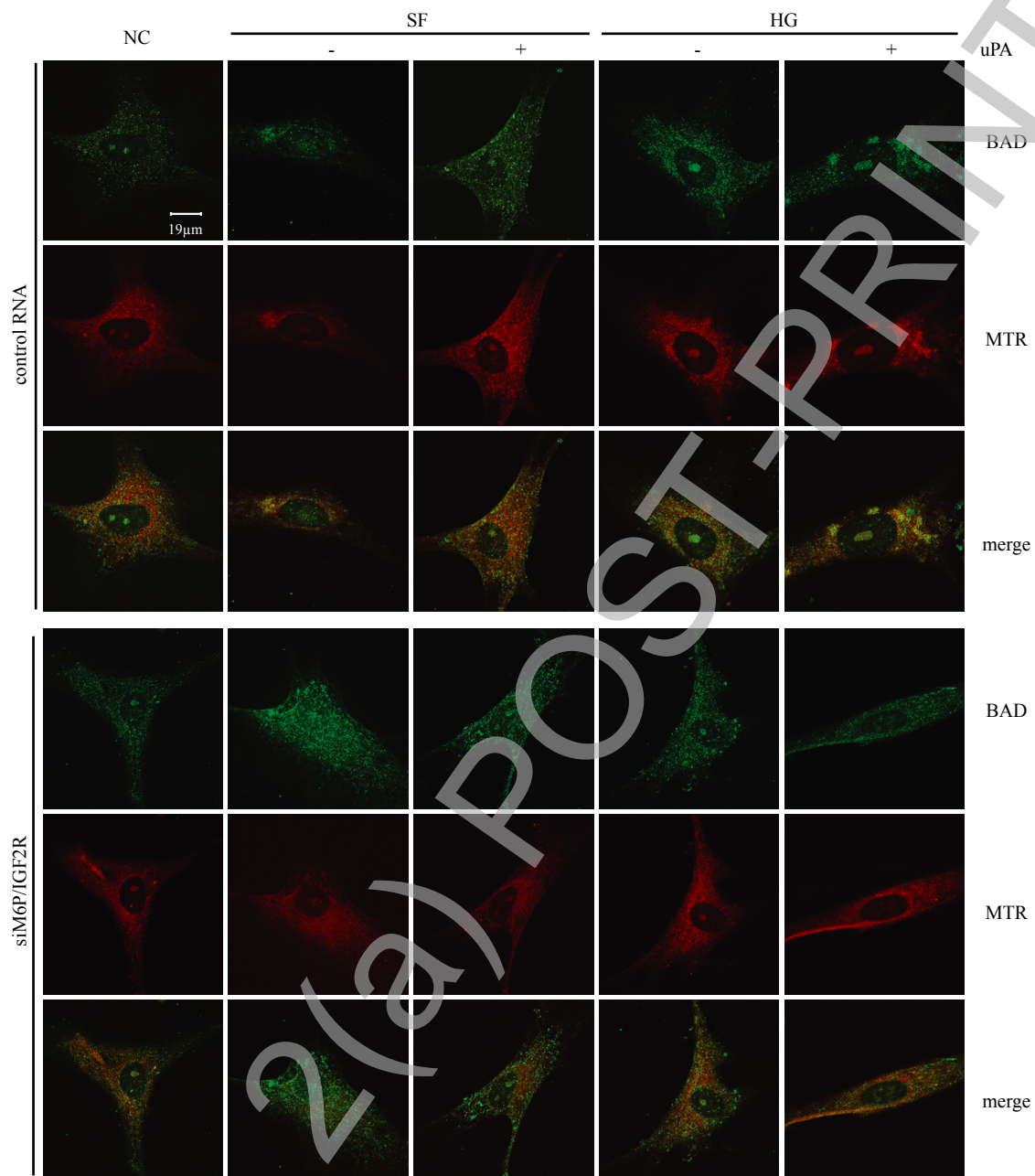


Figure 6



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