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On-Chip, Cell-Based Microarray Immunofluorescence Assay for High-Throughput Analysis of Target Proteins

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We have developed an immunofluorescence-based assay for high-throughput analysis of target proteins on a three-dimensional cellular microarray platform. This process integrates the use of three-dimensional cellular microarrays, which should better mimic the cellular microenvironment, with sensitive immunofluorescence detection and provides quantitative information on cell function. To demonstrate this assay platform, we examined the accumulation of the α subunit of the hypoxia-inducible factor (HIF-1α) after chemical stimulation of human pancreatic tumor cells encapsulated in 3D alginate spots in volumes as low as 60 nL. We also tested the effect of the known dysregulator of HIF-1α, 2-methoxyestradiol (2ME2), on the levels of HIF-1α using a dual microarray stamping technique. This chip-based in situ Western immunoassay protocol was able to provide quantitative information on cell function, namely, the cellular response to hypoxia mimicking conditions and the reduction of HIF-1α levels after cell treatment with 2ME2. This system is the first to enable high-content screening of cellular protein levels on a 3D human cell microarray platform.

Cell-based assays are ideal for screening chemical libraries for molecules that modulate a broad range of biological events. A common approach is to use antibody binding to track specific proteins in the cell;[1] however, such methods are often tedious even in microwell formats and involve multiple steps of reagent addition and washing. Moreover, these techniques are often only qualitative, which limits their value in assessing the influence of small molecules on cellular functions. In this context, microscale technologies are emerging as powerful tools for tissue engineering and biological studies,[2] as well as for drug discovery and human toxicology assays.[3,4] In particular, microarrays have afforded

information from very small sample volumes and have facilitated the incorporation of high-throughput, high-content (e.g., protein-specific) assays into the drug discovery process.[5]

With the advent of robotic spotting technology, it is now possible to distribute nanoliter volumes of different biomolecules and cells in a spatially addressable manner.[6–8] Cell-based microarrays also enable efficient removal of reagents and facilitate subsequent washing steps, thus overcoming common limitations associated with conventional multiwell plate formats.[9] In addition to two-dimensional cell culture arrays, miniaturized three-dimensional (3D) arrays have been developed, which are compatible with high-throughput screening;[4] the more native-like microenvironment of the 3D culture may enhance the quality and biological relevance of data that can be obtained in high-throughput screens.[10] Despite these advances, 3D cellular microarrays for immunoassay-based screens have not been developed.

Here we present a high-throughput, cell-based immunofluorescence microarray screening platform (an in-cell, chip-based Western assay) designed to evaluate specific protein levels inside human cells. Hypoxia-mimicking conditions within human pancreatic tumor cells were studied as a model system, and the α subunit of the hypoxia-inducible factor (HIF-1α) was chosen as the molecular target. Hypoxia occurs in many physiological contexts such as embryonic development,[11] but a dysfunction in cellular oxygen homeostasis is also related to a number of pathophysiological situations, such as angiogenesis in cancer.[12] A primary effector of the adaptive response to hypoxia in mammals is the HIF family,[13–15] in particular the HIF-1α subunit. The

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diminished level of oxygen in a growing tumor leads to the accumulation and activation of the transcription factor HIF-1α, which in turn induces the transcription of a large number of genes involved in tumorigenesis and angiogenesis, including the vascular endothelial growth factor-A responsible for the formation of new blood vessels that migrate into the tumor mass.[12,16] Therefore, the identification of novel small molecules that activate or inhibit HIF-1α activity may be useful for the development of new therapies for ischemic disorders and cancer. In a broader context, this platform can be further extended to other applications such as protein and drug screening, enzyme inhibition, and cytotoxicity assays.

EXPERIMENTAL SECTION

Cell Culture. Upon thawing, Mia PaCa-2 human pancreatic tumor cells (ATCC, Manassas, VA) were grown in T75 tissue culture flasks until confluence at 37 °C in a 5% CO_2 incubator (HEPA class100, Thermo, Waltham, MA), using low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin–streptomycin, and 1% (v/v) MEM nonessential aminoacids, all from Invitrogen (Carlsbad, CA). The cells were either trypsinized and seeded in six-well plates (1×10^5 cells/mL) or used for cell spotting on functionalized glass slides. After each passage, viable and dead cells were determined by counting in a hemocytometer using an optical microscope and the trypan blue dye (Invitrogen) exclusion test.

Western Blotting Analysis of HIF-1α Levels in Mia PaCa-2 Cells. Mia PaCa-2 cells were used to assess the expression of HIF-1α. The cells were seeded in six-well plates (1×10^5 cells/mL) and grown until confluence at 37 °C in a 5% CO_2 incubator. The medium was replaced every 48 h. The hypoxia-mimicking agent carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132, EMD Chemicals, Gibbstown, NJ) was used for induction of HIF-1α expression and 2-methoxyestradiol (2ME2, Sigma, St. Louis, MO) was used for inhibition of HIF-1α.

A whole-cell extract was prepared by lysing the cells with 100 mM potassium phosphate (pH 7.8) and 0.2% (v/v) Triton X-100 supplemented with protease and phosphatase inhibitors. After cell lysis, the lysate was collected and equal amounts of protein were loaded in each lane. The samples were resolved in a 7.5% precast SDS-PAGE gel for 1 h at 100 V and electrotransferred to a cellulose membrane, both from Bio-Rad (Hercules, CA). The membrane was then immersed in blocking buffer (5% (w/v) skim milk and 0.05% (v/v) Tween-20 in phosphate-buffered saline (PBS)) overnight with agitation.

The primary antibody (mouse monoclonal anti-HIF-1α, BD Biosciences, San Jose, CA) was diluted (1:250, v/v) in the blocking buffer and incubated with the membrane for 1 h at room temperature. The membrane was then washed three times for 10 min with 0.05% (v/v) Tween-20 in PBS (PBS-T) and incubated with the secondary antibody (peroxidase-conjugated goat anti-

mouse IgG, Pierce, Rockford, IL) (1:1000 dilution in blocking buffer) overnight at 4 °C. The membrane was again washed three times for 10 min with PBS-T and immunoreactivity was visualized using a chemiluminescence reagent (SuperSignal, Pierce). β-Actin was used as internal control (rabbit monoclonal anti-β-actin, Cell Signaling Technology, Danvers, MA).

Glass Slide Modification. Borosilicate glass slides (25×75 mm^2, Fisher, Pittsburgh, PA) were prewashed with ethanol followed by acid treatment in concentrated sulfuric acid (98%) overnight to remove dust and oil from the glass surface. The slides were then sonicated for 30 min and rinsed in deionized distilled water five times and then once in acetone. The cleaned glass slides were dried using a nitrogen stream and then baked at 120 °C for 15 min prior to use. To generate slides for cell spotting the acid-cleaned glass slides were spin-coated (WS-400B-6NPP/Lite, Laurell Co., North Wales, PA) with 0.1% (w/v) poly (styrene-co-maleic anhydride) (PS-MA, Sigma) in toluene solution as described previously.[4] Briefly, the 0.1% (w/v) PS-MA solution was prepared starting from a 1% (w/v) stock solution, and 1.5 mL was spin-coated on top of a clean glass slide surface at 3000 rpm for 30 s. Methyltrimethoxysilane (MTMOS, Sigma)-coated slides were also prepared as described previously.[3] These slides were used for stamping MG-132 and 2ME2 in the cell spots to perform HIF-1α induction/inhibition studies on the microarrays.

Cell Spotting. A poly-L-lysine (PLL)-Ba^{2+} mixture was prepared by mixing a solution of BaCl$_2$ in water (0.1 M) and sterile PLL (0.01% (w/v)), both from Sigma, in a 1:2 volume ratio, respectively. This mixture was spotted on the PS-MA-coated glass slides using a MicroSys 5100–4SQ noncontact microarray spotter (Genomic Solutions, Ann Arbor, MI). A mixture of low-viscosity alginate (Sigma) and cell suspension in DMEM (supplemented with 10% FBS) was prepared so that the final concentrations of cells and of alginate were 6×10^6 cells/mL and 1% (w/v), respectively. During cell spotting, the humidity within the microarrayer chamber was maintained above 90% to retard water evaporation from the applied spots. To minimize potential microbial contamination, the microarray spotter chamber was sterilized with 70% ethanol prior to use, and growth medium was supplemented with antibiotics (penicillin and streptomycin).

Cellular Viability Assay. The spotted slides were immersed in DMEM/10%FBS complete medium and incubated at 37 °C in a 5% CO_2 incubator for 5 days. Medium was changed after 48 and 96 h. Cellular viability was assessed every day with the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Invitrogen). Briefly, the slides were rinsed three times in PBS and a solution containing 0.5 μM calcein AM was used to detect viable cells through green fluorescence intensity. The fluorescence was scanned and quantified from the scanning image using GenePix Pro 6.0 (Molecular Devices Co., Sunnyvale, CA). The fold increase in total viable cell number was calculated by the ratio of the fluorescence intensity at each day and the fluorescence intensity at day 0.

Mimicking Hypoxia in Cellular Microarray. Spotted slides were immersed in 10% FBS-supplemented DMEM medium and incubated for 3–4 days at 37 °C in a 5% CO_2 incubator. Several dilutions of the hypoxia mimicking agent MG-132 were made by adding this agent to the culture medium, followed by 6-h incubation. The slides were then processed, and the levels of HIF-

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1α were detected using the in situ immunofluorescence assay. Slides incubated under normoxic conditions (no MG-132 added) were used for comparison. To show the potential of this platform for screening small molecules and their effects in cell models, we dispensed 60 nL of known concentrations of MG-132 and 2ME2 onto MTMOS-coated slides. The resulting array was immediately stamped on top of the 3D cellular array, such that each compound-containing spot contacted a cell spot.[4] A silicone gasket was used to maintain proper spacing between the two slides. The dual slide system was subsequently incubated for 6 h at 37 °C in a nearly 100% humidified hybridization chamber (ArrayIt, Sunnyvale, CA) to prevent evaporation. After stamping, the cell spots were rinsed and the cellular microarray was analyzed using the in-cell, chip-based Western assay.

Cellular Microarray-Based Immunofluorescence Assay. Following incubation the slides were rinsed three times in washing buffer (PBS containing 20 mM $CaCl_2$) for at least 15 min, followed by fixation with a solution of methanol and acetone (1:1, v/v) for 20 min at −20 °C. The slides were then rinsed twice in washing buffer and incubated overnight in blocking solution (SuperBlock, Pierce). The primary antibody (mouse anti-HIF-1α, 1:500 dilution in PBS-T containing 1% (w/v) bovine serum albumin (BSA)) was then added to the slides, and the cells were incubated overnight at 4 °C. The slides were then washed three times in washing buffer for 15 min each, the secondary antibody (peroxidase-conjugated goat-anti mouse IgG, Molecular Probes (Carlsbad, CA), 1:1000 dilution in PBS-T containing 1% (w/v) BSA) was added to the slides, and the cells were incubated for 3 h at room temperature. A tyramide signal amplification kit (Molecular Probes) was used following the manufacturer's instructions to detect the presence of the protein HIF-1α through fluorescence analysis. The fluorescence was measured with the slide scanner and the images of stained cells were analyzed using the program GenePix Pro 6.0. The fluorescence signal of β-actin (determined by the same protocol using triplicate slides for each condition) was used as the internal control.

RESULTS AND DISCUSSION

We employed human pancreatic tumor cells, Mia PaCa-2, which are known to overexpress the hypoxia-inducible factor in response to hypoxic conditions.[17] Hypoxic conditions in vivo, particularly those related to angiogenesis, occur within a 3D cellular environment. To achieve a similar situation for the current work, we used a 3D cell-on-a-chip technique involving alginate-based matrixes on a microscope slide that was recently developed in our groups.[4] The glass surface of the slides was functionalized with a reactive copolymer of polystyrene and maleic anhydride (PS-MA; Figure 1a), which increases the surface hydrophobicity and provides reactive groups to covalently attach the highly cationic PLL. A mixture of PLL and $BaCl_2$, used to initiate alginate gelation, was spotted onto defined positions on the PS-MA surface. The formation of 3D spots was achieved by spotting 60 nL of an alginate solution containing the cells onto the PLL/$BaCl_2$ bottom layer; alginate gelation was nearly instantaneous, and the positively charged PLL promoted attachment of the negatively charged polysaccharide constituent of alginate upon gelation. The resulting spots had a diameter of ∼800 μm and a center-to-center distance

between adjacent spots of ∼1200 μm (Figure 1b). This spotting method requires very little reagent for fabrication of the biochip and produces a functional microarray platform for high-throughput studies that is compatible with conventional microarray scanning for quantitative analysis.

To assess the suitability of the microscale 3D alginate matrix for cell growth, an array of 560 spots containing human pancreatic Mia PaCa-2 cells at an initial seeding density of 100 cells/spot was incubated in cell culture medium for 5 days. During this period, the cells remained confined within the spots and no visible gel detachment from the slide was observed, indicating that the spots were structurally stable. Cell viability assays were performed daily by staining a slide with the fluorescent dyes calcein AM and ethidium homodimer-I to quantify live and dead cells, respectively. High levels of live cells were detected after 5 days in culture (Figure 1c), indicating that the hydrogel environment did not seriously affect cellular viability. Live cell numbers per spot correlated linearly with green fluorescence intensity (Figure 1d), which facilitated cell growth calculations. Moreover, the cells reached a (4.7 ± 0.7)-fold increase in total cell number at the end of the 5-day incubation period (Figure 1e). Also shown in Figure 1e are images of representative individual microarray spots. No change in spot diameter was observed, indicating that the alginate matrix was stable throughout time in culture. The calculated apparent specific growth rate was $0.30 ± 0.05$ day^{-1} with a corresponding doubling time of $2.2 ± 0.3$ days; these values are similar to those obtained for Mia PaCa-2 cell growth in six-well plate cultures ($0.37 ± 0.01$ day^{-1} and $1.9 ± 0.04$ days). Mia PaCa-2 cells could thus be cultured in the microarray system without losing viability, and the culture performance was not affected by the near 2000-fold scale reduction from typical 96-well plates.

In order to quantify HIF-1α levels in situ, Mia PaCa-2 cells were treated with serial dilutions of MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) to stimulate the accumulation of HIF-1α. MG-132 is a potent, reversible, and cell-permeable proteasome inhibitor that reduces the degradation of ubiquitin-conjugated proteins in mammalian cells by the 26S complex.[18] Under normoxic conditions, HIF-1α is rapidly degraded by the proteasome; however, the use of proteasome inhibitors has been shown to result in the cellular accumulation of HIF-1α.[19] Western blotting analysis of whole-cell lysates from six-well plates revealed a clear dose-dependent response of HIF-1α accumulation as a result of increasing concentrations of MG-132 (Figure 2). As a control, cellular levels of β-actin were measured. This protein is not under the influence of hypoxic control elements and represents a simple and assayable target that is dependent only on cell viability.[15] Therefore, blockage of the proteasomal degradation machinery by a proteasome inhibitor results in substantial accumulation of HIF-1α.

This immunofluorescent method was scaled down to function at the microscale and allow high-throughput analysis. Such an in-cell, on-chip Western analysis would enable rapid quantification of the levels of specific cell marker proteins on the microarray. The strategy is depicted in Figure 3. Briefly, after fixing the cells, a primary antibody specific to the target protein was added to

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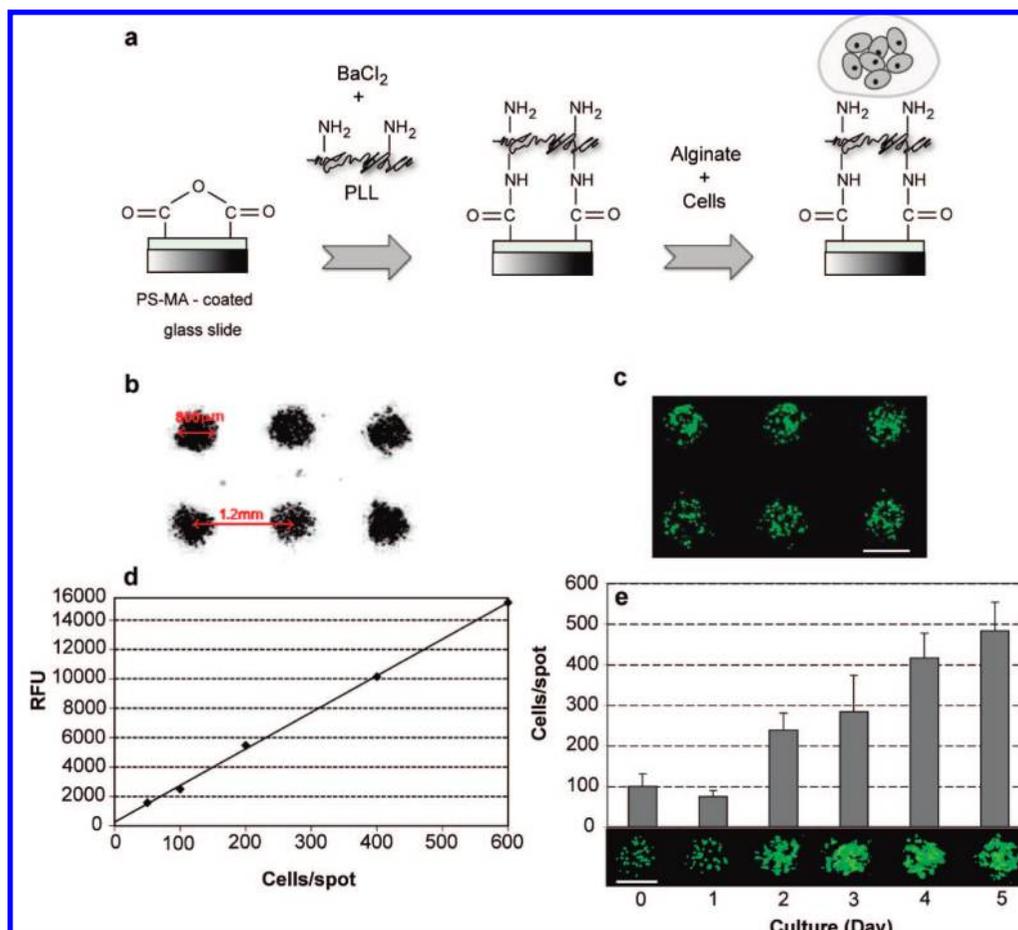
Figure 1. 3D Cellular microarray platform for quantitative analysis of target proteins. (a) Cell encapsulation on a functionalized glass slide. The cells are spotted on PS-MA-modified glass slides using a microarray spotter. (b) Light microscope image of a portion of the cellular microarray. (c) Scanning image of a portion of the cellular microarray. After 5-days growth in the alginate-based microarray spots, Mia PaCa-2 cells exhibited high levels of live cells (scale bar 800 μm). (d) Correlation of intensity (RFU) of calcein AM stain integrated over the spot area with the number of cells present in the spot.(e) Cell growth in the alginate microarray spots. Total viable cell number per spot for Mia PaCa-2 cells grown for 5 days in the 3D alginate spots (60 nL, initial seeding of 100 cells/spot) (scale bar 800 μm).
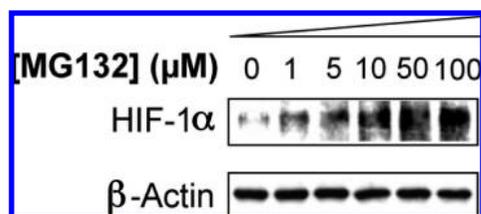
Figure 2. Western blotting analysis of cellular accumulation of HIF-1α after incubation with the proteasomal inhibitor MG-132. β-Actin was used as internal control.

the chip. A strong signal was obtained by adding a horseradish peroxidase (HRP) secondary antibody complex that binds to the primary antibody. The HRP generates multiple copies of a tyramide dye, which is highly reactive and covalently attaches to nucleophilic residues in the vicinity of the HRP–target interaction site, minimizing diffusion-related loss of signal and enabling the quantification of the target protein in each individual spot without spot-to-spot contamination.

The levels of HIF-1α expressed in Mia PaCa-2 cells cultured for 3 days on the microarray platform were measured using the in-cell, on-chip Western protocol following 6-h exposure with MG-132 at concentrations up to 100 μM. As was observed with the
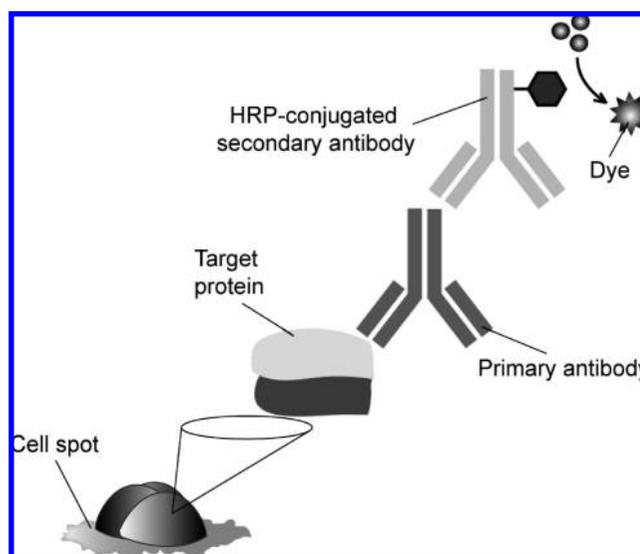
Figure 3. Schematic of the in-cell, on-chip Western technique.

standard Western analysis, a dose-dependent response of HIF-1α accumulation with increasing concentrations of MG-132 was obtained (Figure 4a). To account for spot-to-spot variability, all data were normalized by dividing the signal obtained for HIF-1α
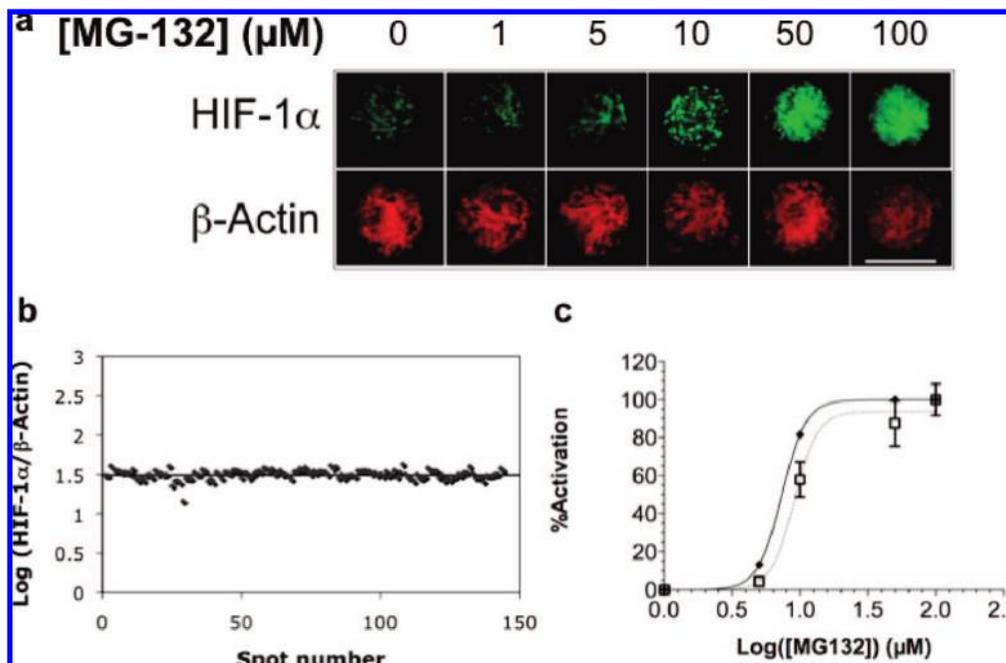
Figure 4. In-cell, on-chip Western analysis of HIF-1α in the microarray platform. (a) Mia PaCa-2 cells were cultured for 3 days in the spots, and the slides were incubated for 6 h with different concentrations of MG-132 (scale bar 800 μm). (b) Response signal measured across a block of 12 × 12 spots under the same conditions (100 μM MG-132 added). (c) Dose−response curves for HIF-1α after treatment with MG-132. The data were normalized such that the minimum and maximum responses corresponded to 0 and 100%, respectively. Curves obtained by standard Western blotting (filled diamonds) and in-cell, on-chip Western analysis (open squares) in the microarray platform are shown for comparison.

(given by the Alexa Fluor 488 dye) by the level of the internal control β-actin (provided by the Alexa Fluor 594 dye). The signal measured across a block of 12 × 12 spots under identical conditions (100 μM MG-132 added) was not influenced by the position on the chip (Figure 4b) or between chips. With respect to the latter, under conditions where 100 μM MG-132 was added (similar to Figure 4b), the ratio of Log (HIF-1α/β-actin) was essentially constant among three independent chips and ranged from 1.50 ± 0.06 to 1.48 ± 0.05. The extent of nonspecific binding of secondary antibody was evaluated by spotting the cells and reproducing the protocol without adding the primary antibody. The signal due to nonspecific binding was <10% of the signal obtained at the lowest concentration of MG-132 applied to the chip. This signal was subtracted from the fluorescence signal obtained for each MG-132 concentration. By taking into account the minimal background signal and using the signal of β-actin as internal control, the accumulation of HIF-1α was monitored in response to increasing concentrations of MG-132. Lower concentrations of MG-132 (<5 μM) did not induce a significant increase in the levels of HIF-1α. However, when compared with basal levels (no MG-132 added), higher concentrations of MG-132 (>5 μM) led to the stabilization of HIF-1α and the consequent accumulation of this protein in the cells. A nearly 8-fold increase in HIF-1α levels was obtained for cells induced at the highest concentration of MG-132 (100 μM) when compared with the signal obtained in the absence of inducer.

To compare our method with standard Western blotting, we calculated the half-maximal effective concentration (EC_{50}) of MG-132 that induces an increase in HIF-1α response. To that end, the data were normalized so that the lowest response corresponded to the baseline and the highest response to the maximum, or 100% activation, and EC_{50} values were calculated

from the sigmoidal dose−response curves (Figure 4c). A chip-based EC_{50} value of 9.1 ± 2.1 μM was calculated based on the in situ quantification of HIF-1α, which is comparable to the EC_{50} value calculated using the standard Western blotting results (EC_{50} = 7.2 ± 0.2 μM). The results on-chip, therefore, were similar to those obtained using the standard Western blotting, which demonstrates that the in-cell, on-chip immunofluorescence protocol serves as a reliable, high-throughput technique to quantify the levels of target proteins in situ.

We also evaluated an on-chip, in-cell immunofluorescence assay to determine loss of function (e.g., inhibition) using both MG-132 and 2ME2. The latter is an endogenous estrogen metabolite that inhibits angiogenesis and endothelial cell proliferation[20] in an oxygen- and proteasome-independent manner.[15] To facilitate the addition of both compounds onto the cell-containing microarray, an MTMOS-coated glass slide was prepared and spotted with specific concentrations of MG-132 and 2ME2. The MTMOS slide is complementary to the 3D cell-containing alginate array. Bringing both slides together via stamping, followed by incubation, provides a dual slide system that enables the small molecules to diffuse into the cell spots and induce a biological response. A similar stamping strategy was shown to be effective in the high-throughput analysis of small molecule cytotoxicity.[4]

The high-throughput capacity of the on-chip, in-cell immunofluorescence assay was then tested using a 14 × 20 array of 60-nL spots printed onto MTMOS-coated slides with MG-132 (100 μM) or with MG-132 (100 μM) plus 2ME2 (100 μM). The printed MTMOS slides were then stamped on top of the 3D cell-containing alginate array (also prepared in a 14 × 20 spot arrangement that was a mirror image of the small-molecule-containing slide), such

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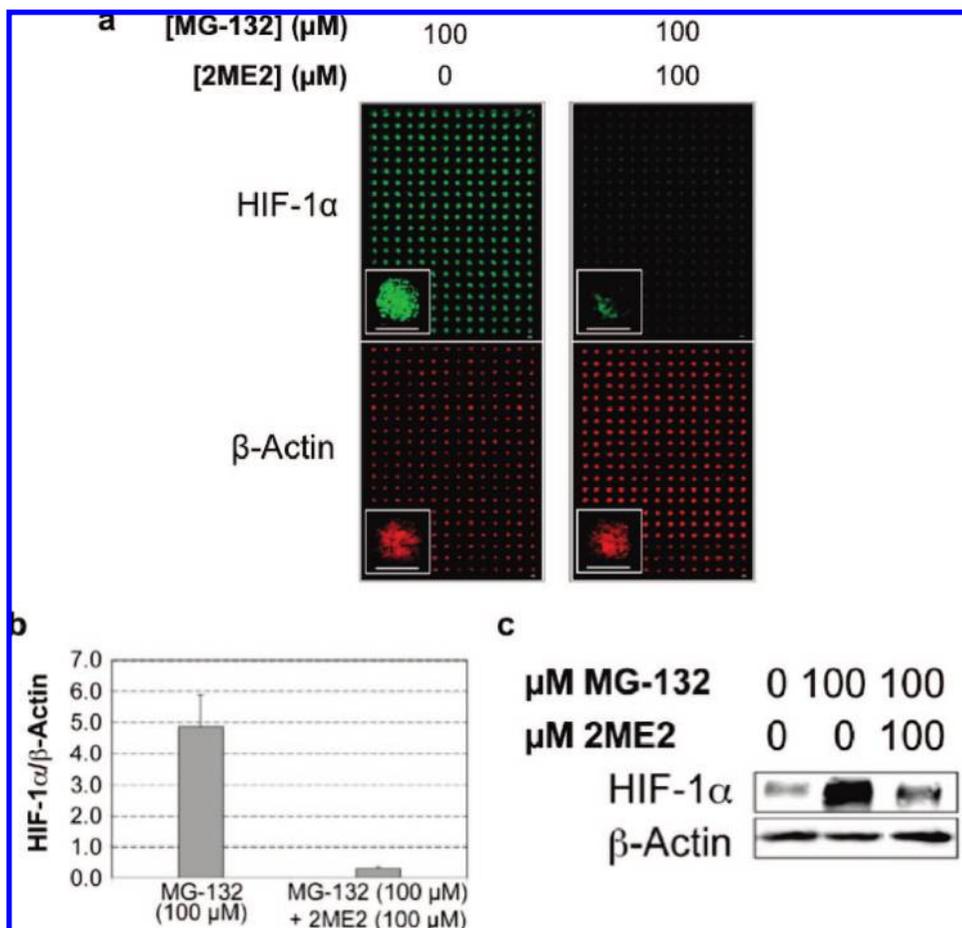
Figure 5. Dysregulation of HIF-1α after incubation with 2ME2. (a) In-cell, on-chip Western analysis of HIF-1α in the microarray platform. Mia PaCa-2 cells were cultured 3 days in the spots, and the slides were stamped with MTMOS-coated slides spotted with MG-132 (100 μM) or MG-132 and 2ME2 (100 μM each). The dual slide system was incubated for 6 h before analysis (scale bar 800 μm).(b) Quantification of HIF-1α levels on the 3D cellular microarray after stamping with 2ME2. The results are presented as the ratio of HIF-1α and β-actin signals for each condition. (c) Western blotting analysis. Mia PaCa-2 cells were cultured until confluence in well plates and incubated for 6 h either with 100 μM MG-132 or MG-132 and 2ME2 (100 μM each). After cell lysis, whole-cell lysates were collected and analyzed. β-Actin was used as internal control.

that each small-molecule spot was in contact with a cell spot, as previously described. The stamped slides were incubated for 6 h, after which the slides were separated and the levels of HIF-1α were measured. As before, β-actin was used as an internal control.

The presence of MG-132 resulted in high levels of HIF-1α expression (Figure 5a). The stamping procedure did not affect the overall stability of the microarray spots or cell viability; the scanned images show uniform spots with the inset indicating a high level of protein expressed by the cells in a representative spot. 2ME2 caused considerable reduction of HIF-1α levels (reduction in green fluorescence in the scanned image in Figure 5a). In neither case (MG-132 with and without 2ME2) did the level of the cellular control β-actin change. These results are consistent with the influence of 2ME2 on cell function. 2ME2 acts by depolymerizing tumor cell microtubules, which results in blockage of HIF-1α nuclear accumulation and HIF-transcriptional activity.[15] As a result, even in the presence of MG-132, posttranscriptional downregulation of HIF-1α would occur. The results in Figure 5b are consistent with the effect of 2ME2, where inhibition was observed even in the presence of 100 μM MG-132. Finally, consistent with such activity, conventional Western blotting analysis of whole-cell lysates (Figure 5c) showed that 2ME2 reduced HIF-1α accumulation even in the presence of high

concentrations of MG-132. These results demonstrate the feasibility of using the in-cell, on-chip immunofluorescence technique to screen small molecules and to evaluate their effects on the expression levels of molecular targets.

CONCLUSIONS

We have developed a microarray-based in-cell immunoassay protocol for the quantification of protein levels in 3D cultured human cells. This approach may be used to evaluate the effects of small molecules on target protein levels. In the case of the hypoxia mimicking agent MG-132, the resulting in-cell, on-chip induction and accumulation of HIF-1α in human pancreatic tumor cells were observed, and changes in HIF-1α levels resulting from the use of different concentrations of MG-132 were assessed. Inhibition of HIF-1α on the microarray platform was also examined through use of the small-molecule inhibitor 2ME2. The results with MG-132 and 2ME2 were in close agreement to those obtained using conventional Western immunoblotting of cells under hypoxic mimicking conditions.[15] The 3D cell microarray is a high-throughput platform that enables quantification of on-chip cellular protein levels following perturbation of the cells upon addition of small molecules. This method should have many applications in

high-content, cell-based screening regimens for the discovery of new therapeutic agents.