Caveolin-1/PTRF upregulation constitutes a mechanism for mediating p53-induced cellular senescence: implications for evidence-based therapy of delayed wound healing in diabetes

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Submitted 12 April 2013; accepted in final form 31 July 2013

Bitar MS, Abdel-Halim SM, Al-Mulla F. Caveolin-1/PTRF upregulation constitutes a mechanism for mediating p53-induced cellular senescence: implications for evidence-based therapy of delayed wound healing in diabetes. Am J Physiol Endocrinol Metab 305: E951–E963, 2013. First published August 13, 2013; doi:10.1152/ajpendo.00189.2013.—A heightened state of oxidative stress and senescence of fibroblasts constitute potential therapeutic targets in nonhealing diabetic wounds. Here, we studied the underlying mechanism mediating diabetes-induced cellular senescence using in vitro cultured dermal fibroblasts and in vivo circular wounds. Our results demonstrated that the total antioxidant capacity and mRNA levels of thioredoxinreductase and glucose-6-phosphate dehydrogenase as well as the ratio of NADPH/NADP were decreased markedly in fibroblasts from patients with type 2 diabetes (DFs). Consistent with this shift in favor of excessive reactive oxygen species, DFs also displayed a significant increase in senescence-associated β-galactosidase activity and phospho-γ-histone H2AX (pH2AX) level. Moreover, the ability of PDGF to promote cell proliferation/migration and regulate the phosphorylation-dependent activation of Akt and ERK1/2 appears to be attenuated as a function of diabetes. Mechanistically, we found that diabetes-induced oxidative stress upregulated caveolin-1 (Cav-1) and PTRF expression, which in turn sequestered Mdm2 away from p53. This process resulted in the activation of a p53/p21-dependent pathway and the induction of premature senescence in DFs. Most of the aforementioned oxidative stress and senescence-based features observed in DFs were recapitulated in a 10-day-old diabetic wound. Intriguingly, we confirmed that the targeted depletion of Cav-1 or PTRF using siRNA or Vivo-Morpholino antisense-based gene therapy markedly inhibited diabetes/oxidative stress-induced premature senescence and also accelerated tissue repair in this disease state. Overall, our data illuminate Cav-1/PTRF-I as a key player of a novel signaling pathway that may link a heightened state of oxidative stress to cellular senescence and impaired wound healing in diabetes.

polymerase I and transcript release factor

WOUND HEALING TYPICALLY PROCEEDS in a highly ordered series of events that encompasses the following four phases: inflammation, granulation, reepithelialization, and tissue remodeling (34). This process appears to be compromised in elderly and diabetic patients and also in patients receiving chemotherapy and/or radiotherapy, which ultimately results in the development of slow or nonhealing ulcers. Impaired wound healing is a growing public health burden, with costs running into billions of dollars annually in the US alone. Indeed, the public health impact of impaired wound healing will likely increase within the coming decades due to the aging world population and the pandemic increase in type 2 diabetes (6).

During the course of wound healing, dermal fibroblasts play dual functions as a synthetic cell that deposits the extracellular matrix and signaling cells that synthesize and secrete the growth factors essential for tissue repair. Normal fibroblasts can typically divide 50–70 times in tissue culture before they become senescent (44); this phenotype is characterized by the enlargement and spreading of the cells, an accumulation of lipofuscin, the expression of senescence-associated β-galactosidase (SA-β-gal), cell cycle arrest in G1, and an increase in polynucleation (7). These cells can be exploited by the tumor microenvironment to limit the progression of certain cancer types, but they may also have a detrimental influence on injured tissue regeneration (29). Cellular senescence can also be induced by stressors [stress-induced premature senescence (SIPS)] such as reactive oxygen species (ROS), of which hydrogen peroxide (HP) is the most common; other known stressors include hyperoxia, ultraviolet light, γ-irradiation (23), and oncogenic stimulation (45, 55). Most of these SIPS-related paradigms are associated with the activation of p53-, p21-, and/or p16-retinoblastoma protein-dependent pathways (13, 42).

Recent data have indicated that cellular senescence represents a major contributing factor in the induction of nonhealing chronic wounds (18). Indeed, cell replicative ability was diminished by ~50% in pressure ulcer fibroblasts compared with adjacent normal fibroblasts (48). Similarly, an increase in fibroblast senescence appears to predominate in the venous ulcers and skin tissue of diabetic mice (37). Consistent with these findings, it was reported that a number of senescence-like features (e.g., the increased expression of SA-β-gal, decreased production of cyclin D1, phosphorylated RB, and growth factors, and increased level of p21) can be recapitulated by exposing normal fibroblasts in culture to wound fluids derived from chronic nonhealing wounds (36, 47). It is noteworthy that the most common features of these chronic wound microenvironments include markedly increased ROS levels, an active species that attacks DNA, causing the accumulation of lipofuscin (a molecule that cannot be degraded by cells) and DNA damage-induced cell cycle arrest (7–9), the decreased expression and secretion of growth factors [epidermal growth factor (EGF), platelet derived growth factor (PDGF) and insulin like growth factor I (IGF-I)], decreased keratinocyte migration, increased tissue proteases, and microbial contamination (1, 12).
A wealth of evidence is now available implicating caveolin-1 (Cav-1), a major resident scaffolding protein constituent of caveola, in the induction of cellular senescence (52, 53). In this vein, old human fibroblasts and replicative senescent mesenchymal stem cells and bone marrow stromal cells have been shown to express higher levels of Cav-1 compared with their control counterparts (56). Moreover, Cav-1 upregulation positively correlates with the reduced replicative lifespan of chondrocytes following treatment with interleukin-1β (54). Consistent with these findings, Cav-1 expression is also elevated in various tissues of old animals, including the lungs, spleens, and brains (28, 38). This involvement of Cav-1 in replicative senescence can be recapitulated when cells or tissues are exposed to excessive levels of oxidative stress (56). For example, it was shown that a sublethal HP level increases endogenous Cav-1 expression and induces premature senescence in NIH 3T3 cells (51). Interestingly, this HP-induced premature senescence was greatly inhibited in NIH-3T3 cells receiving antioxidants (51) or harboring antisense Cav-1 (4) and in mouse embryonic fibroblasts derived from Cav-1 null mice, which do not express Cav-1 (4). Taken together, these data suggest a central role for Cav-1 in the signaling events that regulate oxidative stress-induced premature senescence.

In light of the above data and our recent reports (16, 17) showing that dermal fibroblasts and wounded tissues from rats with type 2 diabetes exhibit increased ROS generation and decreased antioxidant capacity, a novel hypothesis was formulated, stating that cellular senescence stemming from the oxidative stress–mediated overexpression of Cav-1 constitutes a mechanism for the development of nonhealing diabetic ulcers. As an initial step toward supporting this proposition, we assessed cellular senescence and key molecules in the signaling pathway of Cav-1 as a function of diabetes using in vitro and in vivo models of wound healing. Here, we identify Cav-1 as a novel signaling player that links oxidative stress to the impairment of wound healing in diabetes.

**MATERIALS AND METHODS**

**Cell culture and HP treatment.** Primary dermal fibroblast cell lines were established from the dorsal skin of the female Goto-Kakizaki (GK; age 12–15 mo) rat, a model for nonobese type 2 diabetes (DFs) and their Wistar control counterparts (CFs), as described previously (9). Briefly, the dermis was cut into small pieces and incubated in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) containing collagenase (250 U/ml; Sigma) for 30 min at 37°C in 5% CO₂ with constant agitation. The sections were triturated vigorously to release fibroblasts, and these cells were collected by centrifugation. The resulting pellet was washed twice with phosphate-buffered saline (PBS), resuspended in complete medium [DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), 2 mM glutamine, and 10 mM HEPES] and then cultured under standard conditions.

**Oxidatively stressed CFs.** A heightened state of oxidative stress and premature senescence in CFs were induced, using HP as a stressor. The HP treatment was conducted by seeding ~1 × 10⁶ cells in a T75 tissue culture flask. Twenty-four hours later the cells were exposed to a sublethal dose of HP (150 μM) for 2 h and washed twice with PBS, and after 4 days of culturing in HP-free medium they were split in a 1:2 ratio, and then the aforementioned protocol was repeated again for cells receiving the second and third HP treatment. Third-passage HP-treated CFs, denoted in the current study as oxidatively stressed CFs (OS-CFs), together with the corresponding CFs and DFs were used in most of the assays outlined below.

**SA-β-gal staining and quantification assay.** The SA-β-gal activity was determined using a senescence β-gal staining kit (Cell Signaling Technology). The experiments were performed in six-well plates, following the instructions of the manufacturer. Cells were washed three times with PBS, fixed for 3 min at room temperature in 3% formaldehyde, washed, and then incubated overnight at 37°C without CO₂ in a freshly prepared staining medium [1 mg/ml 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal), 40 mM citric acid-sodium phosphate, pH 6.0, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl₂] (22). Cells were then examined for blue-green staining of cytoplasm indicative of senescence. For a precise quantification of β-gal activity, the rate of conversion of 4-methylumbelliferyl-β-d-galactopyranoside (MUG) to the fluorescence-hydrolyzed product 4-methylumbelliferone (4-MU) at pH 6 was assessed using previously published procedure (25).

**pH2AX immunofluorescence staining and quantification assay.** Phosphorylation of H2AX at Ser¹³⁹ (pH2AX), a sensitive marker for cellular senescence and DNA damage, was evaluated using either an immunofluorescence staining or a fluorometric-based assay kit (Active Motif). Briefly, cells derived from various experimental groups were cultured on coverslips for 24 h and then washed (PBS), fixed (4% paraformaldehyde, 10 min), permeabilized (0.3% Triton X-100, 10 min), blocked (2% BSA, 1 h), and incubated with H2AX phosphorylated at Ser¹³⁹ overnight at 4°C. After the samples were rinsed with PBS, the coverslips were incubated for 1 h in the dark with the secondary goat anti-rabbit conjugated to Texas Red (Invitrogen). Samples were mounted with Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories). Immunofluorescence microscopy was performed using a Zeiss Confocal microscope (Carl Zeiss). A fluorometric-based assay kit (Active Motif) was used to quantitatively assess the level of pH2AX. Cells or wounded tissues were sonicated in SDS lysis buffer, and the DNA fragments were confirmed to be within 100–1,000 bp. About 5 μg/ml anti-pH2AX was added to the lysates and allowed to incubate at 4°C overnight. Thereafter, the samples were washed and incubated for 1 h at room temperature with Alexa 488 goat anti-rabbit antibody, and then the fluorescence was measured at ~485-nm excitation and 520-nm emission wavelengths using a fluorometer (Promega).

**Growth rates.** Control and diabetic fibroblasts were seeded at densities of 1 × 10⁴ and 3 × 10⁴ cells/ml, respectively, in DMEM supplemented with 10% FCS and antibiotics. Seeding at these concentrations gave ~50% confluence for both cell types. The cells were harvested at confluence (N₀), and their population doublings (PDLs) were estimated using the previously published formula (RCPD) = log₂(Nf/N₀)/time in days, Nf is the number of cells at the end of each passage of cell growth.

**Cell cycle analysis and synchronization.** Cells at 70–80% confluence were washed with PBS, detached with a trypsin-EDTA solution, and fixed with 70% ethanol for 30 min. After washing with PBS, the fixed cells were incubated with RNAase A for 30 min at 37°C, resuspended in 0.5 ml PBS, stained with propidium iodide in the dark for 30 min, and then analyzed using a FACScan equipped with a Xe/Ne laser at 488 nm.

To promote cell cycle synchronization, the cells were rendered quiescent by serum deprivation for 48 h, and this step was followed by the addition of PDGF to stimulate the cells to reenter the cell cycle. The cell populations in the G1, S, and G/M phases were quantified with PBS, fixed for 3 min at room temperature in 3% formaldehyde, washed, and then incubated overnight at 37°C without CO₂ in a freshly prepared staining medium [1 mg/ml 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal), 40 mM citric acid-sodium phosphate, pH 6.0, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl₂] (22). Cells were then examined for blue-green staining of cytoplasm indicative of senescence. For a precise quantification of β-gal activity, the rate of conversion of 4-methylumbelliferyl-β-d-galactopyranoside (MUG) to the fluorescence-hydrolyzed product 4-methylumbelliferone (4-MU) at pH 6 was assessed using previously published procedure (25).

**Assessment of key fibroblast functions essential for wound healing.** To assess cell proliferation, fibroblasts were seeded into a 96-well plate at a density of 1 × 10⁴/well and were allowed to adhere overnight in DMEM medium supplemented with 10% FCS. After

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arrest by incubation in serum-free medium for 24 h, the cells were exposed to growth factors, and the incorporation of bromodeoxyuridine (BrdU) into DNA was determined by following the manufacturer’s protocol (Roche Diagnostics). Similarly, for the in vitro wound (migration) experiments, cultured fibroblasts were grown in six-well plates until they reached confluence. The medium was removed, and the cells were washed with PBS three times before culturing was continued in serum-free DMEM containing 0.5% BSA for an additional 24 h. Thereafter, the monolayer was wounded artificially by using a pipette tip to scratch across the plate; the cells were washed with PBS to remove the detached cells and then cultured in serum-free medium in the presence of mitomycin C (10 μg/ml) to prevent cell proliferation. The rate of wound healing was quantified according to our previously published procedure (2).

Apoptotic cell death. Cytoplasmic levels of cytchrome c (Onco-gene), histone-associated DNA fragments (mono- and oligonucleosomes; Roche), and caspase 3 activity (Clontech, USA) were determined according to the manufacturer’s instructions. The total antioxidant capacity (TAC) in fibroblasts and wounded tissues was assessed by Trolox equivalent antioxidant capacity assays. Total RNA from cells or wounded tissues was sonicated on ice in RIPA buffer containing 1% NP-40, 0.5% deoxycholate, and a protease/phosphatase inhibitor cocktail (Roche Diagnostics), and the resulting homogenates were centrifuged at 15,000 g for 15 min at 4°C. The protein concentrations in the supernatants were determined by the BCA Protein Assay (Pierce). For immunoprecipitation, 500 μg of the supernatant protein was incubated with 20 μl of protein A-G (Santa Cruz Biotechnology) and 5 μg of antibody overnight at 4°C under constant rotation. Nonspecific IgG was used as a negative control. Immunoprecipitates were washed twice with RIPA buffer before the addition of 2X Laemmli buffer. Proteins derived from the total lysates and immunoprecipitates were loaded onto an SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked and then incubated with the primary antibody diluted in 5% nonfat dry milk in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) overnight at 4°C. The blots were incubated with the secondary antibody conjugated to HRP in TBST for 1 h at room temperature. The proteins were visualized with the Super Signal West PicoChemiluminescent Substrate (Pierce) according to the manufacturer’s protocol.

Real-time PCR for mRNA quantitation. Total RNA from cells or frozen wounded tissues was extracted using the Trizol reagent (Invitrogen), and the integrity of the RNA was verified using agarose gel electrophoresis. Approximately 1 μg of RNA was reverse transcribed (Superscript II Reverse Transcriptase Kit; Invitrogen) and amplified using the TaqMan Assay on Demand (Applied Biosystems) in a 25-μl reaction volume containing two unlabeled primers, a 6-carboxyfluorescien-labeled TaqMan MGB probe and the master mix. The amplified sequences were assessed using the ABI 7500 Prism Sequence Detection system machine. The results were expressed as mRNA levels normalized to 18S or GAPDH in each sample.

siRNA/cDNA transfection. The expression of Cav-1 and polymerase I and transcript release factor (PTRF) in fibroblasts of type 2 diabetes was abolished by siRNA oligonucleotides. The sequences were designed and synthesized by Qigang. The day before transfection, cells were seeded at a density of 1.75 × 10² cells/well in a six-well plate in complete DMEM medium. The next day, cells were washed once with OptiMEM medium (Gibco) and then overlaid with 800 μl of OptiMEM medium. Optimum silencing efficiency was obtained by adding 15 μl of 20 μM siRNA to 145 μl of 37°C OptiMEM medium and incubated at room temperature for 15 min, and then Oligofectamine mixture (Invitrogen) was added according to the manufacturer’s instructions (8 μl Oligofectamine + 32 μl of OptiMEM medium, incubated for 5 min at room temperature). Complexes were added to the cells, and after 4 h of incubation in the CO₂ incubator, 500 μl of DMEM containing 30% FCS was added to the well. Following overnight incubation, the cells were washed with PBS and incubated in DMEM. This process was conducted every 3 days for 9 days.

As for overexpression of Cav-1, pCMV6 (vector) and pCav-1 (plasmid that overexpresses rat Cav-1) were purchased from OriGene Technologies, and Lipofectamine 2000 (Invitrogen) was used for the transfection. Efficiencies of the knockdown and overexpression of Cav-1, PTRF, or Keap1 were verified by real-time PCR or Western blotting.

Wound model, drug treatment, and macroscopic evaluation. All of the animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Female GK rats were used in the current study. Detailed information regarding this animal model for nonobese type 2 diabetes has been described in our previous publications (10, 11). Weight- and age-matched female Wistar rats (Kuwait University breeding colony) served as the corresponding controls. All of the animals were maintained under standard conditions with a 12-h on/off light cycle, commercial diet, and water ad libitum. GK rats destined for wounded were initially matched with regard to body weight (e.g., 250–300 g) and plasma levels of glucose, free fatty acids, and insulin. These indices are commonly used to reflect the severity of the diabetic state.

The animals were anesthetized by intraperitoneal injection of 90 mg of ketamine + 10 mg xylazine/kg body wt, and their back skin was shaved, depilated with Nair, and cleaned with 70% alcohol. Six bilateral full-thickness excisional wounds (8 mm in diameter) equidistant from the midline were created on the dorso-rostral back skin. The wounds were separated by a minimum of 1 cm of uninjured skin. The targeted depletion of Cav-1 was achieved using the antisense vivo-Morpholino oligomers (MOs) that were synthesized and designed by Gene Tools (Eugene, OR). Antisense or 4-mismatch negative control MOs at 75 μM were applied to the wound in a vehicle of pluronic acid in PBS solution. The specificity of the Cav-1 morphant phenotype was determined using rat Cav-1 cRNA that lacked the antisense MO binding site. The efficiency of the knockdown was confirmed using a Western blotting-based technique. The wounds were photographed at various time intervals after injury (0-, 1-, 3-, 5-, 7-, 10-, and 14-day periods; please note only the 10-day period is shown) using a Sony n-9 digital camera. The wound area was analyzed using Adobe Photoshop (version 7.0; Adobe Systems), and the percentage of wound closure was derived by the following formula: (1 – [current wound sizes/initial wound size]) × 100. A maximum effect of the vivo-Morpholino Cav-1 antisense-based therapy was observed macroscopically on day 10 during the course of wound healing. Accordingly, this time point was selected and used in the study of the various parameters related to Cav-1 signaling, pro-oxidant/antioxidant capacity, and the biochemical markers of cellular senescence. It is noteworthy that previous studies have indicated that the 7- to 10-day period is a critical time not only for healing (39, 40) but also for growth factor-based activity (5, 27).

Statistical analysis. The data are expressed as means ± SE. A one-way analysis of variance with Bonferroni post hoc validation or the Mann-Whitney test was used to compare the data derived from various experimental groups. A level of P ≤ 0.05 was considered to be significant.
RESULTS

Accumulation of senescent cells in cultured fibroblasts of type 2 diabetes. Previous studies from our laboratory have demonstrated that cultured fibroblasts of type 2 diabetes (DFs) are associated with a heightened state of oxidative stress, exemplified by increased ROS generation and decreased antioxidant capacity (8, 9). This phenomenon is known to induce premature senescence in primary cells and tissues (23). Accordingly, we aimed to determine whether cellular senescence is a characteristic feature of the diabetic state. Our data revealed that DFs showed a marked enhancement in SA-β-gal activity at pH 6 assessed either by the positively blue-stained cells (Fig. 1, Aa and Ab) or the rate of conversion of MUG to 4-MU (Fig. 1Ac), increased DNA damage foci accompanied by
histone H2AX phosphorylation (Fig. 1, Ba and Bb), and growth inhibition as exemplified by the decrease in both the rate of cell doubling per day (Fig. 1C) and BrdU incorporation into DNA (Fig. 1D). Moreover, the expression of p53 (Fig. 1E) and p21 (Fig. 1F), key regulators of cellular senescence, was also upregulated in DFs compared with their control counterparts. Interestingly, these phenotypic features of senescence in DFs were associated with an aberration in the systems that are involved in oxidative biosynthesis (NADPH/NADP⁺), maintenance of the cellular redox balance, and ROS scavenging [e.g., mRNA levels of glucose-6-phosphate dehydrogenase (G6PD) and thioredoxinreductase; Fig. 1G]. Consistent with these data, we also confirmed in DFs a significant reduction in total antioxidant capacity in situ, which was quantified using a Trollex-based assay (Fig. 1H).

To gain insight into whether the decreased resistance to oxidative stress could explain the premature onset of cellular senescence in DFs, we compared the tendency of these cells and their control counterparts to undergo changes in senescence-based biomarkers in response to a sublethal dose (150 μM) of hydrogen peroxide (HP), a well-known model of oxidative stress-mediated premature senescence. During the first cycle of HP treatment, DFs exhibited an increase in SA-β-gal activity and the level of pH2AX at PDL doubling 35, a phenomenon that was not apparent in CFs (data not shown). Similarly, the aforementioned paradigm also negatively affected the redox/antioxidant network in diabetic but not control fibroblasts. It is noteworthy that CFs exposed to a paradigm of chronic oxidative stress (OS-CFs) recapitulated most of the senescence-based features observed in DFs (Fig. 2, A–F). Together, these data support the premise that DFs with an altered redox balance and increased oxidative damage have a higher propensity for stress-induced cellular senescence. The above findings regarding DFs and OS-CFs are reminiscent of those reported previously in G6PD- and Gpx1-deficient fibroblasts (16, 21).

Cav-1 overexpression contributes to cellular senescence in DFs. An accumulation of senescent cells may arise from the increased expression of factors controlling oxidative stress-mediated cellular senescence, such as CCN1 (CYR61), plasminogen activator inhibitor-1, or Cav-1 (26, 52, 53, 56). Cav-1-mediated cellular senescence, such as CCN1 (CYR61), plasminogen activator inhibitor-1, or Cav-1 (26, 52, 53, 56). Cav-1 has been shown to play a key role in replicative and stress-induced premature senescence (SIPS) (53, 56). A sublethal dose of HP increased endogenous Cav-1 expression and induced premature senescence in NIH-3T3 cells (51). As indicated above, DFs are more sensitive to HP and exhibit a state of heightened oxidative stress. Accordingly, we examined the Cav-1 signaling pathway in the context of diabetes and cellular senescence. The data derived from these studies confirmed that the Cav-1 level was markedly increased in DFs compared with their control counterparts (Fig. 2A). This diabetes-induced Cav-1 upregulation was apparently associated with impaired PDGF ability to regulate the phosphorylation-dependent activation of Akt (Fig. 2B) and ERK1/2 (Fig. 2C). Other growth-promoting polypeptides, including IGF-I and EGF, also exhibited in DFs a pattern of effect similar to that seen with the PDGF (data not shown). Our data are consistent with previous reports indicating that Cav-1 may bind to and negatively regulate EGF and PDGF receptors (19).

Further experiments were conducted to examine the impact of the attenuation in growth factor signaling on key fibroblast functions essential for wound healing, including cell proliferation and cell migration. The proliferation index, as exemplified by the incorporation of BrdU and the cell cycle profile, was assessed by exposing starved cultured fibroblasts to well-known mitotic growth factors or to 10% serum. Because the stimulation potency of these factors varies, a dose response ELISA for BrdU incorporation was established for each factor, and the dose providing the maximum response was selected. Figure 2D shows that, in serum-induced proliferating cells in CFs, the optical density at 450 nm was >10-fold higher than that observed for starved cells. Similarly, PDGF, EGF, and IGF also increased the proliferation rate ~10-, 7.1-, and 6.7-fold, respectively (Fig. 2D). This stimulatory action of the growth factors on cell proliferation was markedly suppressed in diabetic conditions (Fig. 2D). To further dissect the mechanisms underlying growth rate inhibition, the cell cycle profile was analyzed using a flow cytometry-based technique. We found that in response to PDGF, DFs showed lowered propensity for S phase entry and an increased number of cells arrested in G0/G1 compared with corresponding control values (Fig. 2E).

Cell migration is an additional parameter related to tissue regeneration, and this process was examined in cultured fibroblasts in response to a mechanical wound. Fibroblasts in monolayer culture were subjected to a mechanical scratch wound injury model in the presence or absence of PDGF, IGF-I, and EGF or serum. The number of cells that migrated into the cell-free wound zone over the course of 24 h postinjury in CFs was markedly increased in response to these growth factors, with PDGF being the most potent among them (data are shown for PDGF only; Fig. 2F). This phenomenon was reduced by ~50% in DFs compared with the corresponding control values (Fig. 2F).
Intriguingly, the unresponsiveness of DFs to the aforementioned growth-promoting polypeptides in terms of cell signaling and cell proliferation/migration was recapitulated in CFs subjected to a chronic paradigm of oxidative stress (e.g., OS-CFs; Fig. 2, A–F).

Further studies viewed DFs and OS-CFs in the context of apoptotic cell death, a well-known companion of cellular senescence. In this regard, we confirmed that the levels of cytoplasmic cytochrome c, histone-associated DNA fragments (mono- and oligonucleosomes), and the activity of caspase 3/7 were increased by ~47 ± 5.8, 55 ± 7.2, and 43 ± 6.1%, respectively, in DFs relative to CFs. A similar observation was also evident in OS-CFs. Taken together, our data harmonize with the concept that, at least in diabetes and in the heightened state of oxidative stress, cellular senescence appears to be associated with a marked enhancement in the level of apoptosis in addition to an attenuation in the actions of growth-promoting polypeptides.

Gain-of-function and loss-of-function strategies in control and type 2 diabetic fibroblasts. To examine whether a cause-and-effect relationship exists between the overexpression of Cav-1 and the phenotypic features of diabetes-induced cellular senescence, we instituted a loss- and gain-of-function genetic strategy. In this regard, we showed that CFs overexpressing Cav-1 (CFs-pCMV-Cav-1) exhibited a marked increase in the activity of $\alpha$-SA-gal (Fig. 3A) and the levels of $\alpha$2X (Fig. 3B), p53 (Fig. 3C), and p21 (Fig. 3D) compared with their corresponding vector-treated (CFs-pCMV-GRP) control values. *Significantly different from the corresponding control values at $P \leq 0.05$. 

Fig. 2. DFs and OS-CFs exhibited a significant increase in caveolin-1 (Cav-1) expression and attenuation in growth factor actions. CF ($n = 3$), DF ($n = 4$), or OS-CF (e.g., control fibroblasts exposed to hydrogen peroxide, $150 \mu$M for 3 passages; $n = 4$) fibroblasts were used to assess Cav-1 expression and the actions of growth factors. Cav-1 protein expression ($A$) and platelet-derived growth factor (PDGF)-induced phosphorylation of Akt ($B$; 20 min) and ERK ($C$; 10 min) in 24-h serum-starved fibroblasts were determined using a Western blotting-based technique. A proliferation index encompassing BrdU incorporation into DNA ($D$) and cell cycle progression ($E$) was evaluated in response to various growth factors [e.g., IGF, 50 ng; PDGF, 1 nM; epidermal growth factor (EGF), 100 ng] in a 24-h serum-starved fibroblast. $F$: an in vitro wound-healing model conducted by scratching starved confluent cultured fibroblasts with a pipette tip was used to monitor the impact of PDGF on cell migration. Most of the assays were performed in triplicate, and the data are expressed as means ± SE of at least 3 independent experiments. *Significantly different from the corresponding control values at $P \leq 0.05$. 

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ues. In contrast, the activity of Akt (Fig. 3E) and the rates of cell proliferation (Fig. 3F) and migration (Fig. 3G) in response to PDGF were attenuated at a high cellular Cav-1 level (Fig. 3, E–G). A subset of the above data has been reported previously in human diploid fibroblasts (38) and in mouse embryonic fibroblasts (51).

We next sought to determine whether knocking down Cav-1 with siRNA in DFs could exert a beneficial effect with respect to cellular senescence and the defect in PDGF actions. Interestingly, the tendency of the diabetic state to induce premature senescence, as reflected by the increase in SA-β-gal activity (Fig. 3A) and the levels of pH2AX (Fig. 3B), p53 (Fig. 3C),...
and p21 (Fig. 3D), appeared to be ameliorated in cells harboring the Cav-1 siRNA (Fig. 3, A–D). Consistent with these findings, we also showed that reducing Cav-1 level in DFs using the siRNA-based technique partially restored PDGF responses, as exemplified by the increased phosphorylation of p-Akt (Fig. 3E) and the enhanced rates of cellular proliferation (e.g., DNA synthesis; Fig. 3F) and migration (Fig. 3G). Overall, our data support the notion that cellular senescence (e.g., level/activity of SA-β-gal, pH2AX, p53, and p21) and decreased rates of cell proliferation and migration in DFs may stem at least in part from Cav-1 overexpression. More intriguingly, a functional recovery of DFs in terms of growth factor responsiveness appears to be attainable by instituting a therapeutic strategy that reduces the Cav-1 level. In view of the fact that Cav-1 is required at the cross-talk of various signaling transduction pathways as a scaffold protein, it becomes essential to determine the optimum degree of reduction of diabetic wound Cav-1 level to minimize the negative impact of this intervention upon other potential signaling cascades. This issue is now under consideration in our laboratory.

Cav-1 overexpression in DFs induces cellular senescence through a mechanism involving Mdm2/PTRF-dependent signaling. To investigate the molecular mechanism(s) underlying cellular senescence and the activation of p53-p21 signaling as a function of diabetes, we focused on the PTRF/Cav-1/Mdm2-dependent pathway. This is not unreasonable since both PTRF and Cav-1 are colocalized within the caveolae, are involved in the sequestration of Mdm2 (an ubiquitin ligase that targets p53 for degradation), and have been shown to be elevated during replicative senescence and oxidative stress-induced cellular senescence (3, 49). Our initial data showed that the protein expression of PTRF was markedly increased in DFs compared with their corresponding control values (Fig. 4A). We then asked whether diabetes, in addition to upregulating PTRF protein expression, could also increase the interaction between PTRF and Cav-1. Coimmunoprecipitation studies in DFs demonstrated a significant enhancement in the binding of PTRF to Cav-1 (Fig. 4B) and also of Cav-1 to Mdm2 (Fig. 4C). In contrast, the binding affinity of p53 for Mdm2 was markedly suppressed as a function of diabetes (Fig. 4D). The current findings suggest a sequence of events in which a heightened state of oxidative stress in DFs upregulates PTRF/cavin-1 protein expression, which in turn promotes the membrane localization of Mdm2 and its interaction with the already overexpressed Cav-1. This increase in the sequestration of Mdm2 by Cav-1 in DFs activates the p53/p21-dependent pathway with the concomitant induction of cellular senescence. Support for the aforementioned proposition is best exemplified by our data documenting that PTRF downregulation in DFs via an siRNA-based strategy reduced not only the levels of SA-β-gal and pH2AX (Fig. 4E) but also the level of p53 (Fig. 4F). Moreover, partial restoration of PDGF activity, viewed in the context of cell proliferation/migration (Fig. 4G) and Akt phosphorylation, was also evident in the siRNA/PTRF/DFs (Fig. 4H). It is noteworthy that most of the data regarding the changes in PTRF/Cav-1 dynamics are not unique to the diabetic state, since similar findings were confirmed in OS-CF cell line (Fig. 4, A–H). A case in point in this regard is our unpublished observation confirming that the premature senescence in DFs/OS-CFs can also be reversed by counteracting the heightened state of oxidative stress using the novel Nrf2 activator glycyrrhetinic acid.

An in vivo-Morpholino-based knockdown of Cav-1 ameliorated both premature senescence and impaired wound healing in GK diabetic animals. Emerging data have indicated that cellular senescence and an increased ROS level may contribute to impaired wound healing under various pathological conditions (8, 9, 46). Consistent with these findings, the overexpression of Cav-1 has been shown to inhibit muscle repair mechanisms in cell culture studies and in vivo (33, 50), whereas accelerated skin wound healing has been reported in Cav-1 null mice (33). The current data and those reported previously by our laboratory clearly demonstrate that a heightened state of oxidative stress, the overexpression of Cav-1, and cellular senescence are characteristic features of DFs, a key cell that is involved in the reparative mechanism of the healing process (8, 9). To gain insights that are pathologically and clinically relevant, we assessed the effect of the diabetic state on key biomarkers of cellular senescence and oxidative stress as well as the interaction between Cav-1/PTRF and Mdm2 over the course of cutaneous wound healing. Our data documented that activities of SA-β-gal and pH2AX were enhanced in a 10-day-old diabetic wounds (Fig. 5A). Similarly, the levels of key molecules within the Cav-1-dependent signaling, including Cav-1 (Fig. 5B), PTRF (Fig. C), p53 (Fig. 5D), and p21 (Fig. 5E), were also upregulated as a function of diabetes. Consistent with the aforementioned abnormalities, we also showed that diabetic wounds displayed a significant alteration in Cav-1 dynamics, exemplified here by the increase in the binding of Cav-1 to Mdm2 (Fig. 5F) and PTRF (Fig. 5G), in addition to a reduction in the interaction between Mdm2 and p53 (Fig. 5H).

We then wondered whether a heightened state of oxidative stress, the constant companion of senescence, represents a characteristic feature of a 10-day diabetic wound. This phenomenon was assessed by determining key molecules in the prooxidant/antioxidant network, with the resulting data confirming that NADPH oxidase activity and the mRNA level of its subunit NOX1 were enhanced (Fig. 5I) in the 10-day diabetic wound. Contrastingly, the in situ total antioxidant capacity (Fig. 5J), the ratio of NADPH to NADP, and the mRNA levels of expression of antioxidant enzymes (e.g., TR, G6PD) were reduced as a function of diabetes (Fig. 5K). In aggregates, our findings harmonize with the premise that chronic oxidative stress activates the Cav-1-dependent signaling, and this in turn contributes at least in part to the senescence-based features of nonhealing diabetic wounds.

Next, to prove that a cause-and-effect relationship indeed exists between the upregulation of Cav-1-dependent signaling, cellular senescence, and impaired wound healing in diabetes, we applied the so-called vivo-Morpholino Cav1-based antisense to a diabetic circular skin wound, using pluronic acid as a vehicle. This novel wound-based knockdown strategy ameliorated the diabetes-related increase in wound contents of SA-β-gal/pH2AX (Fig. 5A), Cav-1 (Fig. 5B), PTRF (Fig. 5C), p53 (Fig. 5D), and p21 (Fig. 5E). Moreover, the enhanced binding of Cav-1 to Mdm2 (Fig. 5F) and PTRF (Fig. 5G) and the decreased affinity of Mdm2 for p53 (Fig. 5H) in a 10-day diabetic wound was also restored almost to normal values in response to the vivo-Morpholino treatment. The above beneficial effect of Cav-1 knockdown in diabetic wounds was not
limited to the senescence and Cav-1 signaling pathway but extended also to the heightened state of oxidative stress. In this connection, we showed that total antioxidant capacity (Fig. 5J), the ratio of NADPH/NADP, and the level of expression of G6PD and thioredoxinreductase (TR; Fig. 5K) were increased in MO-D (diabetic wounds treated with Morpholino Cav1-based antisense) compared with corresponding vehicle-treated diabetic wounds. These data regarding the antioxidant capacity are of interest, especially when viewed in the context of the recent findings showing that overexpression of Cav-1, like that of diabetes, suppressed the activity of Nrf2 and its associated phase 2-dependent antioxidant enzymes (8, 32). A case in point in this regard is our data confirming that a number of the phenotypic features of premature senescence (e.g., PDGF-induced cell proliferation, SA-β-gal activity) in DFs can also be reversed by counteracting the heightened state of oxidative stress, using a pharmacological approach typified by the novel Nrf2 activator glycyrrhetinic acid 18α-GA or a genetic-based paradigm involving the selective siRNA-based knockdown of Keap1, a cytoskeleton-associated protein that when complexed with Nrf2 promotes its ubiquitin- and proteasomal-mediated degradation. In this regard, we have shown that the SA-β-gal activity was reduced by about 42 ± 5.8 and 47 ± 6.3% in 18α-GA and siRNA Keap1-treated DFs, respectively, compared with vehicle-treated DFs. Similarly, these treatments also ameliorated diabetes-related deficits in the proliferative action of PDGF (CFs = 1 ± 0.15, DFs = 0.43 ± 0.048, DFs/18α-GA = 0.71 ± 0.087, DFs/siRNA/Keap1 = 0.77 ± 0.093). The most intriguing and novel finding,
however, lies in the fact that delayed healing in diabetic wounds can be ameliorated by a genetic knockdown of Cav-1 (Fig. 5L). Overall, the current study is consistent with the notion that the overexpression of Cav-1, possibly stemming from a state of heightened oxidative stress, contributes at least in part to the underlying mechanisms of cellular senescence and impaired wound healing in diabetes.

**DISCUSSION**

Cellular senescence constitutes a mechanism that inhibits mammalian cell growth in response to damage or stress. This process plays a crucial role in age-related diseases and tumorigenesis and appears to be associated with accumulated DNA damage, a limited number of cell divisions, and a reduced

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**Figure Legends**

A. Graph showing fold change vs control for SA-β-gal and PH2AX. B. Western blot for Cav-1/Actin with bands for 1-C, 2-D, and 3-MO-D. C. Western blot for PTRF/Actin with bands for 1-C, 2-D, and 3-MO-D. D. Western blot for p53/Actin with bands for 1-C, 2-D, and 3-MO-D. E. Western blot for p21/Actin with bands for 1-C, 2-D, and 3-MO-D. F. Western blot for IP:Mdm2/IB:Cav-1 with bands for 1-C, 2-D, and 3-MO-D. G. Western blot for IP:Cav-1/IB:PTRF with bands for 1-C, 2-D, and 3-MO-D. H. Western blot for IP:Mdm2/IB:p53 with bands for 1-C, 2-D, and 3-MO-D. I. Graph showing fold change vs control for NADPH oxidase (Activity) and NOX-1 (mRNA). J. Graph showing TAC (mM Trolox/mgprotein) for C, D, and MO-D. K. Graph showing fold change vs control for G6PD (mRNA), TR (mRNA), and NADPH/NADP (Ratio). L. Bar graph showing % Wound closure for C, D, and MO-D post wounding (10 days).
Caveolin-1 has recently been identified as a novel gene that regulates replicative senescence and SIPS (56). However, the function of Cav-1 in the progression of cellular senescence and impaired wound healing in diabetes has not been described previously to the best of our knowledge. The current study demonstrated that fibroblasts of type 2 diabetes display senescence features when grown in culture. In this context, DFs exhibited a number of senescence-based features, including the increased expression of SA-β-gal, a reduced rate of growth/DNA synthesis, and hyporesponsiveness to the mitotic effect of factors and serum. These findings indicate that reduced resistance to ROS may lead to oxidative damage and eventually to growth arrest and cellular senescence. The aforementioned proposition is consistent with our current results and previous reports by our laboratory documenting that DFs accumulate large amounts of oxidative byproducts, such as pH2AX/8-OHdG/protein-bound carbonyls, in addition to the increased activity/expression of NADPH oxidase, a severe exhaustion of the intracellular antioxidant defenses, and ultimately more senescent cells in response to repetitive HP treatment (8, 9). It is noteworthy that the heightened state of oxidative stress and cellular senescence in DFs are apparently associated with a marked enhancement in the p53/p21 tumor suppressor pathway. One possible mechanism that may link oxidative stress to cellular senescence in diabetes has been illuminated by the assessment of key components of caveolae, including Cav-1, Mdm2, and PTRF.

Cav-1 is the major structural component of caveolae, and it appears to exert a variety of biological functions, including the regulation of cholesterol levels, vascular transport, proliferation, and apoptosis in a variety of cell types (52). Recently, this molecule has been implicated as a modulator of oxidative stress and cellular senescence. In this context, the overexpression of Cav-1 protein promotes G1 arrest and premature senescence by a p53/p21-dependent mechanism (51). Our data confirm that Cav-1 protein expression was upregulated in DFs. If Cav-1 overexpression is a key element in promoting cellular senescence during diabetes, DFs harboring Cav-1 siRNA should display phenotypic features that are similar to their control counterparts. Indeed, the current findings demonstrating that Cav-1 siRNA-treated DFs expressed a significantly lower level of SA-β-gal and p53/p21 and also showed an increased rate of proliferation/migration in response to growth-promoting polypeptides support the above proposition. Further experiments were conducted to delineate the manner by which increased Cav-1 expression in DFs activates p53/p21 signaling, a principle instigator of SIPS. To this end, we viewed the aforementioned key players of cellular senescence in diabetes in the context of Mdm2, a negative p53 regulator and a target molecule for the Ca-1 binding motif of p53 (4, 31). Although the total Mdm2 protein level was not altered in DFs, the degree of binding to Cav-1, which was assessed using communoprecipitation, was higher in DFs compared with their corresponding control values. These findings are consistent with previous reports demonstrating that during oxidative stress-induced senescence, Mdm2 is neutralized by Cav-1, thus leading to p53 stabilization (4). Overall, our data and those from previous studies support the hypothesis that Cav-1 upregulation induced by the heightened state of oxidative stress in diabetes seques ters an increased amount of Mdm2. As a result, a decrease in the proteosomal degradation of p53 together with the concomitant activation of p21 (a downstream target of p53 that is responsible for cell cycle arrest) may ensue, culminating in the induction of premature senescence in DFs. Interestingly, we found that p53 stabilization, p21 upregulation, and the induc-

Fig. 5. In vivo-Morpholino-based knockdown of Cav-1 ameliorated both premature senescence and impaired wound healing in type 2 diabetes. Full-thickness excisional wounds were induced in the control and Goto-Kakizaki (GK, a non-obese genetic model of type 2 diabetes) rats, and key parameters related to cellular senescence, Cav-1 signaling, and oxidative stress were measured 10 days postinjury. A: SA-β-gal activity and pH2AX contents were measured using fluorescence-based assay kits. B–E: a protein expression of Cav-1 (B), PTRF (C), p53 (D), and p21 (E) in a 10-day wound was determined by Western blotting. A coimmunoprecipitation/Western blotting-based technique was used to assess the binding of Cav-1 to Mdm2 (F) or PTRF (G) as well as the affinity of Mdm2 for p53 (H). I–K: the pro-oxidant (I) and antioxidant (J and K) capacities in a 10-day wound were quantified using spectrophotometry, ELISA, and TaqMan real-time PCR. L: the rate of healing was determined microscopically, as described in MATERIALS AND METHODS. An in vivo antisense morpholino-based Cav-1 administered with dextran acid was used to negate the diabetes-induced elevation in Cav-1 wound contents. A–L: assessment of parameters related to cellular senescence (SA-β-gal activity and pH2AX contents); A, Cav-1 signaling [protein expression of Cav-1 (B), PTRF (C), p53 (D), and p21 (E) in a 10-day wound was determined by Western blotting. A coimmunoprecipitation/Western blotting-based technique was used to assess the binding of Cav-1 to Mdm2 (F), PTRF (G), or the affinity of Mdm2 for p53 (H)]; pro-oxidants (NADPH oxidase activity and NOX-1 mRNA level; J), antioxidants [total antioxidant capacity (K) and G6PD, TR mRNA levels, and NADPH/NADP ratios (K)], and the rate of healing (L) were performed using the 10-day control, diabetic, and vivo-Morpholino-treated diabetic wounds (MO-D). Most of the assays were performed in triplicate, and the data are expressed as means ± SE of at least 8 animals/group. *Significantly different from the corresponding control values at P ≤ 0.05; **significantly different from the corresponding values of diabetic wounds that received the scrambled vivo-Morpholino at P ≤ 0.05.
tion of premature senescence were markedly inhibited in DFs that had been treated with anti-Cav-1 siRNA.

Although Cav-1 is known to be a key player in SIPS induction, little information is known regarding the upstream molecules regulating this scaffolding protein in diabetes. In a separate set of experiments, inhibition of p38 but not phosphatidylinositol 3-kinase/Akt, JNK, or the ERK1/2-dependent pathway abolished the diabetes-induced Sp1-mediated upregulation of Cav-1 protein expression and SIP development (unpublished observation). More importantly, we also identified a significant elevation in the level of p-p38 under basal conditions and in response to HP treatment. These data are reminiscent of an earlier report confirming that the activation of p38 by a sublethal dose of HP induced premature senescence in normal cells but not in cells lacking Cav-1 (20). Moreover, the p38 MAPK-dependent increased phosphorylation of Cav-1 on tyrosine 14 in response to oxidative stress further supports the idea that this MAPK may regulate Cav-1 at the transcriptional and posttranslational levels (17).

Cav-1, being abundant in almost every cell type in wounds, may play a crucial role in the pathogenesis of impaired wound healing in diabetes. Nonhealing diabetic ulcers are a major cause of patient morbidity. Based on our findings in fibroblasts, which are considered to be the repair-committed cells, we sought to view wound healing impairment in diabetics in the context of Cav-1 signaling and cellular senescence. The data revealed that as in DFs, the Cav-1 level, the degree of Cav-1 binding to Mdm2/PTRF, and the activities of SA-β-gal, pH2AX, and the p53/p21-dependent pathways were all increased in a 10-day-old circular wound in diabetic rats. In contrast, the binding of Mdm2 to p53 appears to be suppressed as a function of diabetes. Intriguingly and potentially important therapeutically, we confirmed that most of the aforementioned abnormalities were ameliorated partially or fully by the application of the vivo-Morpholino/Cav-1-based antisense. In aggregate, these data suggest that a cause-and-effect relationship may exist between Cav-1 signaling, cellular senescence, and impaired wound healing in diabetes. Consistent with this notion, other researchers have reported that the wound-healing capacity is compromised in elderly individuals who have higher Cav-1, p53, and p21 levels (41). Furthermore, overexpression of Cav-1 in corneal epithelial cells or CFs (results of the current study) recapitulated many of the senescence-based features that are manifested in DFs (41). In contrast, accelerated skin wound healing was reported in Cav-1-null mice (33) and in the db/db mouse treated with the p38 inhibitor SCIO-469 (35).

Overall, our data and those of previous studies indicate that Cav-1 is a key player of a novel signaling pathway that links a heightened state of oxidative stress to cellular senescence and impaired wound healing in diabetes. In this model, we proposed that ROS induce the premature senescence of fibroblasts and possibly other cells within the wound (e.g., endothelial cells, epithelial cells, and macrophages) in a Cav-1-dependent manner and that these senescent cells contribute to the pathogenesis of nonhealing diabetic wounds. To this end, one may envision a therapeutic intervention aimed at reducing Cav-1 expression within the wound microenvironment to treat or prevent development of chronic, nonhealing ulcers. However, because Cav-1 is considered to be a tumor suppressor signal in certain forms of cancer, we cannot rule out the possibility that the indiscriminate downregulation of Cav-1 expression may accelerate the healing process but increase the chance of tumor development. Accordingly, a targeted knock down of Cav-1 expression as performed in the current study may represent a future therapeutic strategy in the management of diabetic ulcers.

ACKNOWLEDGMENTS

We thank Anees Fatima Beema and Waleed Al-Ali for their excellent technical support. Moreover, we are also grateful to the staffs at the Health Science Center, Kuwait University, Research Core Facility, for their services. We also thank the late Prof. Mahmood Abdulrahheem Ali (1948–2013) from the Kuwait Foundation for Advancement of Sciences for his valuable support during the course of this work.

GRANTS

This work was supported by the Kuwait Foundation for Advancement of Sciences, Grant No. 2003-130-201.

DISCLOSURES

M. Bitar and F. Al-Mulla filed a patent entitled “Use of Caveolin-1 for the Diagnosis, Management, and Treatment of Human Diabetes and Related Complications, in Particular Non-Healing Wounds” with the US Patent Office through the Litman Law. The authors declare no other financial or other conflicts of interest.

AUTHOR CONTRIBUTIONS

M.S.B., S.M.A.-H., and F.A.-M. contributed to the conception and design of the research; M.S.B. performed the experiments; M.S.B. analyzed the data; M.S.B. interpreted the results of the experiments; M.S.B. prepared the figures; M.S.B. drafted the manuscript; M.S.B., S.M.A.-H., and F.A.-M. edited and revised the manuscript; M.S.B., S.M.A.-H., and F.A.-M. approved the final version of the manuscript.

REFERENCES
