Impact of ER stress-regulated ATF4/p16 signaling on the premature senescence of renal tubular epithelial cells in diabetic nephropathy

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PROMISE SENCEENCE, AS A cellular stress response and cell senescence of renal tubular epithelial cells in diabetic nephropathy. Am J Physiol Cell Physiol 308: C621–C630, 2015. First published January 7, 2015; doi:10.1152/ajpcell.00096.2014.—Premature senescence is an important event during diabetic nephropathy (DN) progression. Here, we investigated the role of endoplasmic reticulum (ER) stress-regulated activation of transcription factor 4 (ATF4)/p16 signaling in the premature senescence of renal tubular epithelial cells (RTECs) during DN development. In the renal tissues of Type 2 DN patients, we detected an increased number of senescent cells; elevated deposition of advanced glycation end products (AGEs); upregulated expression of ER stress marker, glucose-regulated protein 78; as well as overexpression of ATF4 and p16. Similarly, these phenomena were also observed in cultured mouse RTECs following AGE treatment. Interestingly, AGE-induced p16 expression and premature senescence were successfully attenuated by ER stress inhibitor and ATF4 gene silencing. Moreover, AGE-induced premature senescence was mimicked by ER stress inducers and ATF4 overexpression, while suppressed by p16 gene silencing. In addition, ER stress inducers can augment ATF4 expression. Therefore, our results demonstrate that the ER stress-regulated ATF4/p16 pathway is involved in the premature senescence of RTECs during DN progression.

ADVANCED GLYCATION END PRODUCTS; PREMATURE SENCEENCE; ENDOPRIMALIC RETICULUM STRESS; ACTIVATING TRANSCRIPTION FACTOR 4; P16

AGEs on promoting premature senescence of proximal tubular epithelial cells (PTECs) (22). However, the precise mechanism remains unclear.

Activating transcription factor 4 (ATF4) is a basic leucine zipper (blip) transcription factor of ER stress. ER stress-induced ATF4 expression adversely affects the expression of a variety of genes that are involved in cell proliferation, differentiation, metastasis, and autophagy (2). Increased expression of ATF4 facilitates the progression of age-related diseases by modulating cell cycle inhibitors, such as p21, and p27 (3, 10, 11). ATF4 is also implicated in cellular senescence of tumor cells through cell cycle inhibitor p16-dependent and independent pathways (13, 21). Furthermore, p16 is strongly associated with aging in kidneys of humans and rodents (19, 24). We therefore hypothesized that AGEs activate ER stress-regulated ATF4/p16 signaling and induce premature senescence of RTECs, thereby promoting tubulointerstitial fibrosis during DN development.

RESEARCH DESIGN AND METHODS

Patients. Twenty patients with Type 2 DN were recruited from the Department of Nephrology of Daping Hospital, the Third Military Medical University from February 2009 to December 2010. The enrollment criteria were as follows: patients were 55–75 yr old who had a history of Type 2 diabetes, the level of 24 h urine protein was between 500 and 5,000 mg, and DN was diagnosed by pathological renal biopsy (32). Patients who had fever, obvious infections, or high uric acid were excluded. All subjects used insulin to control blood glucose, angiotensin antagonists and calcium channel blockers to control blood pressure, and statins to control lipid levels. No use of traditional Chinese medicine or a sulfonylurea within 3 mo following renal biopsy was allowed.

The renal biopsy was performed for clinical diagnostic purpose to rule out other possible diseases. In cases in which the patients were presenting with nephrotic syndromes and their diabetes history was not significant for explaining the symptoms, renal biopsy was performed. Normal tissues were obtained from nephrectomy for renal hamartoma. The DN group was divided into mild-moderate DN group (urinary protein excretion 500–2,000 mg/day or serum creatinine ≤ 120 μmol/l) and severe DN group (urinary protein excretion > 2,000 mg/day or serum creatinine > 120 μmol/l) according to 24 h urine protein and serum creatinine level. The protocol for this study was approved by the Ethical and Protocol Review Committee of the Third Military Medical University (no. 2011CQ-553), and informed consent was obtained from each patient.
Biochemical analyses. Blood and urine samples were collected one day prior to renal biopsies for biochemical analyses and functional tests. Serum total and low-density lipoprotein (LDL) cholesterol, serum creatinine concentration (SCR), urine creatinine (UCr), HbA1c, and 24 h urinary protein were measured. Estimated glomerular filtration rate (eGFR) was calculated by the CKD-EPI formula.

Senescence-associated-β-galactosidase staining. Senescence-associated-β-galactosidase (SA-β-gal)-positive cells were detected in frozen tissues by the bright cytoplasmic blue precipitate, according to previously described methods (9).

Immunohistochemistry. Tissue specimens were cut into 2–4 cm thick sections. After deparaffinization, rehydration, and antigen retrieval, the slides were blocked with 10% goat serum for 15 min and incubated with primary antibodies at 4°C overnight. The anti-ATF4 (1:50), anti-p16 (1:100), anti-E-cadherin, and anti-glucose-regulated protein 78 (GRP78, 1:400) antibodies were purchased from Millipore (Billerica, MA) or Santa Cruz Biotechnology (Santa Cruz, CA). After a wash with PBS, IgG-conjugated horseradish peroxidase (HRP; Biyuntian, Shanghai, China) and 3,3-diaminobenzidine tetrahydrochloride were employed to visualize the positive signal.

For immunofluorescence staining, tissue sections or cultured cells were blocked with 10% goat serum for 15 min, followed by incubation with first antibodies indicated above at 4°C overnight. After being rinsed with PBS, the samples were incubated with fluorescent isothiocyanate- or Cy3-conjugated IgG (Biyuntian, Shanghai, China) and 3,3-diaminobenzidine tetrahydrochloride were employed to visualize the positive signal. For immunofluorescence staining, tissue sections or cultured cells were blocked with 10% goat serum for 15 min, followed by incubation with first antibodies indicated above at 4°C overnight. After being rinsed with PBS, the samples were incubated with fluorescent isothiocyanate- or Cy3-conjugated IgG (Biyuntian, Shanghai, China) and 3,3-diaminobenzidine tetrahydrochloride were employed to visualize the positive signal.

Cell culture and treatment. Mouse primary RTECs were isolated from the C57/BL6 mice (3–5 days old) as previously described (5). Cytokeratin staining was used to confirm the purity of isolated RTECs. The second passage of RTECs was isolated from the C57/BL6 mice (3–5 days old) as previously described (9).

Table 1. Clinical characteristics of patients with DN and controls

<table>
<thead>
<tr>
<th>Subjects, n</th>
<th>Normal Controls</th>
<th>Mild-Moderate DN</th>
<th>Severe DN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>8</td>
<td>3</td>
<td>14</td>
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<tr>
<td>Sex, men/women</td>
<td>4/4</td>
<td>3/3</td>
<td>8/6</td>
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<tr>
<td>SBP, mmHg</td>
<td>106.3 ± 10.1</td>
<td>133.9 ± 9.8*</td>
<td>151.9 ± 16.6*</td>
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<td>DBP, mmHg</td>
<td>76.8 ± 3.8</td>
<td>85.4 ± 4.6*</td>
<td>91.7 ± 6.9*</td>
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<tr>
<td>Serum creatinine, μmol/l</td>
<td>67.8 ± 9.2</td>
<td>82.3 ± 15.7*</td>
<td>119.5 ± 35.6†</td>
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<tr>
<td>eGFR, ml/min per 1.73 m²</td>
<td>96.2 ± 11.2</td>
<td>79.4 ± 9.6*</td>
<td>57.1 ± 6.6†</td>
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<tr>
<td>Total cholesterol, mg/dl</td>
<td>169.4 ± 8.1</td>
<td>174.8 ± 11.6</td>
<td>196.1 ± 18.2*</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dl</td>
<td>107.4 ± 7.3</td>
<td>112.3 ± 9.2</td>
<td>122.5 ± 14.2*</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>6.73 ± 1.40*</td>
<td>6.73 ± 1.40*</td>
<td>13.09 ± 2.72†</td>
</tr>
<tr>
<td>Urinary protein excretion, mg/day</td>
<td>&lt;150</td>
<td>1030.8 ± 228.7*</td>
<td>3428.0 ± 1403.7†</td>
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<td>UUA/G, U/mmol</td>
<td>0.17 ± 0.02</td>
<td>1.24 ± 0.82*</td>
<td>3.45 ± 1.52*</td>
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<td>Mesangial area/glomerular area, %</td>
<td>10.7 ± 1.4</td>
<td>13.1 ± 3.7*</td>
<td>13.6 ± 4.2*</td>
</tr>
<tr>
<td>GBM thickness, mm</td>
<td>354 ± 51</td>
<td>404 ± 63*</td>
<td>454 ± 72*</td>
</tr>
</tbody>
</table>

DN, diabetic nephropathy; SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; GBM, glomerular basement membrane. *P < 0.05 significant difference vs. normal controls; †P < 0.01 significant difference vs. mild-moderate DN.
Fig. 1. Advanced glycation end-products (AGEs) induce premature senescence of renal tubular epithelial cells (RTECs) by a p16-dependent mechanism. A, B: the coexpression of AGEs and p16 were evaluated by immunohistochemical staining (magnification ×200); the AGEs and p16 colocalized proximal tubular epithelial cells (PTECs) in diabetic nephropathy (DN) patients were significantly higher compared with in control group. DAPI, 4',6-diamidino-2-phenylindole. ***P<0.01. C, D: the coexpression of p16 and E-cadherin was evaluated by immunohistochemical staining (magnification ×200); E-cadherin and p16 colocalized PTECs in DN patients were significantly higher compared with control group. ***P<0.01. E, F: RTECs were treated with 0.75, 1.5, 7.5 μM AGE-BSA or BSA for 48 h. Western blot shows p16 expression increased in a dose-dependent manner; **P<0.05; ***P<0.01. G and H: RTECs were treated with 7.5 μM AGE-BSA or BSA for 12, 24, and 48 h. Western blot shows that p16 expression increased in a time-dependent manner; **P<0.05; ***P<0.01. I–K: PTECs were transfected with control siRNA (100 nm) or p16 siRNA (100 nm) for 24 h and then incubated with AGE-BSA (7.5 mM) or vehicle for 48 h. Ratios of senescence-associated-β-galactosidase (SA-β-gal)- and senescence-associated heterochromatin focus (SAHF)-positive cells and cells in the G0/G1 phase were calculated. **P<0.05.
Cell cycle analyses. For cell cycle analysis, cells (1 × 10⁶/sample) were washed twice with cold PBS and fixed with 70% alcohol in PBS for 12 h at 4°C. Cells were then washed twice with PBS and stained with propidium iodide (100 μg/ml) for 30–60 min at 4°C followed by fluorescence-activated cell sorting analysis. The data were analyzed with CellFIT Cell Cycle Analysis software Version 2.01.2 (BD Biosciences, Franklin Lakes, NJ). The percentage of cells in each cell cycle phase was calculated.

Real-time quantitative PCR. Total RNA from pTECs was extracted with Trizol (Invitrogen, Carlsbad, CA). We used 1 μg of total RNA to synthesize cDNAs with the ImProm reverse-transcription kit (Takara Bio, Shiga, Japan). Finally, SYBR Green PCR Master Mix (TaKaRa Bio Group) was used to perform PCR amplification in an iCycler system (Bio-Rad, Hercules, CA). Relative expression was calculated by the 2−ΔΔCt method with values normalized to the reference gene β-actin.

Western blot. Total protein was extracted with radioimmunoprecipitation assay lysis buffer (Pierce Biochemicals, Rockford, IL). Approximately 40–100 μg protein was separated on 10% SDS-PAGE and transferred to PVDF membranes. Following blocking, membranes were incubated with primary antibodies: GRP78 (1:200), ATF4 (1:200), p16 (1:200), or β-actin (1:500) overnight at 4°C. The membranes were then washed and incubated with secondary HRP-conjugated antibodies (1:1,000, Biyuntian) for 1 h at room temperature. Specific bands were detected using the ECL system (Amersham Biosciences, Piscataway, NJ) and Quantity One software.

Statistical analyses. Statistical analyses were performed with SPSS 13.0 software (IBM SPSS, Armonk, NY). Data are presented as means ± SD. The relationship between the two sets of variables was determined by linear correlation analyses. Others were analyzed with a Student’s t-test or ANOVA, wherever appropriate. Differences were considered significant when P < 0.05.

RESULTS

AGEs induce premature senescence of RTECs by a p16-dependent mechanism. The clinical, biochemical, and pathological characteristics of Type 2 DN patients and non-DN patients are demonstrated in Table 1. The levels of SCR, eGFR, HbA1c, urinary protein excretion, and UAGEs/UCr are obviously higher in Type 2 DN patients compared with those of the control group. The histological features of glomerular membrane thickness, mesangial matrix expansion, and Kimmelstiel-Wilson nodules are significant in DN patients. Expression of p16 and AGE was simultaneously colocalized in the AGE-positive cells (Fig. 1, A and B).

Interestingly, E-cadherin was also expressed in p16-positive cells (Fig. 1, C and D), indicating that the majority of the aging cells were renal tubular cells. In vitro studies also revealed upregulated p16 expression following AGE-BSA incubation; this effect was time and dose dependent, and the peak effect was detected at 7.5 μM after 48 h of AGE-BSA incubation (Fig. 1, E–H).

To further illustrate the roles of p16 in AGE-induced premature senescence, we introduced p16-siRNA into RTECs. The transfection efficiency was 86.44 ± 6.21%. Apparently, p16-siRNA introduction significantly inhibited both p16 mRNA and protein levels (data not shown). p16-siRNA considerably reduced the ratio of SA-β-gal- and SAHF-positive RTECs as well as the proportion of RTECs in the G0/G1 phase in the AGE-BSA-treated group (P < 0.05; Fig. 1, I–K).

ER stress mediates AGEs-induced p16 expression and premature senescence of RTECs. Next, we investigated the roles of ER stress during DN progression. As shown in Fig. 2, the expression of GRP78 and p16 was colocalized in the same pTECs (Fig. 2, A and B), implying a close correlation of ER stress and p16 expression in the pathogenesis of DN. In vitro studies further demonstrated that GRP78 expression was significantly increased at 12 h following AGE-BSA stimulation, which was earlier than the upregulation of p16 and SA-β-gal-positive cells. This increase was also dose and time dependent, and the peak value was detected at 48 h after 7.5 μM of AGE-BSA treatment (Fig. 2, C–F). Moreover, the GRP78-positive cells and p16-positive cells were located in the same SAHF-positive premature cells (Fig. 2, G and H). The effect of ER stress inhibitor 4-PBA (29) on AGE-induced p16 expression and premature senescence was then tested as well. As shown in Fig. 2, I–K, 4-PBA also significantly reduced the ratio of SA-β-gal- to SAHF-positive pTECs, as well as the proportion of pTECs in G0/G1 phase in AGE-BSA-treated groups (P < 0.05). ATF4 upregulates p16 expression in ER stress-mediated premature senescence of RTECs. To further evaluate the role of ATF4 in ER stress-mediated premature senescence of RTECs, we tested whether activation of ATF4/p16 signaling could directly induce premature senescence. To this end, pAdTrack-ATF4 was introduced to RTECs. The transfection efficiency was 96.78 ± 9.64%, and both ATF4 mRNA and protein levels were increased. Notably, pAdTrack-ATF4 overexpression induced p16 expression in both mRNA and protein (P < 0.05, Fig. 3, A–C). ATF4 and p16 expression was colocalized in the same SAHF-positive cell nuclei following pAdTrack-ATF4 transfection (Fig. 3, D and E). Furthermore, pAdTrack-ATF4 overexpression induced premature senescence, as indicated by increased SA-β-gal- and SAHF-positive staining of RTECs (P < 0.05, Fig. 3F). We also assessed the effect of p16 gene silencing on ATF4 overexpression-induced...
Fig. 3. Activation of transcription factor 4 (ATF4)/p16 signaling activation induces the premature senescence of RTECs. A–C: RTECs were infected with pAdTrack-Vector (50 MOI) and pAdTrack-ATF4 (50 MOI) for 48 h. Western blot and quantitative PCR showed increased p16 protein and p16 mRNA levels in the ATF4 overexpression group. ***P < 0.01. D, E: Immunofluorescence shows that ATF4 and p16 were colocated in the same SAHF-positive nucleus after pAdTrack-ATF4 transfection. F–I: RTECs were transfected with control siRNA (100 nM) or p16 siRNA (100 nM) for 24 h, and then cells were cultured with pAdTrack-Vector (50 MOI) or pAdTrack-ATF4 (50 MOI) for 48 h. The ratio of SA-β-gal-positive cells, SAHF-positive cells, and cells in G0/G1 phase were significantly reduced in the p16 siRNA- or ATF4 siRNA-transfected group compared with those in tunicamycin (TM) or thapsigargin (TG) groups. **P < 0.05. Results are from representative experiments performed in triplicate.
premature senescence. ATF4 or p16 expression in RTECs was dramatically increased by pAdTrack-ATF4 transfection; however, the latter was obviously inhibited by p16 siRNA. Moreover, introduction of p16 siRNA resulted in a significant reduction of premature senescence induced by ATF4 introduction (P < 0.05, Fig. 3, G–I).

To further explore the correlation of ATGF4/p16 signaling and ER stress-mediated premature senescence, we treated PTECs with ER stress inducers TM and TG (34, 37). We found that both ATF4 and p16 expression was extensively increased at 48 h following TM or TG stimulation, and the highest effect was detected at 72 h (P < 0.01, data not shown). Colocalization of ATF4 and p16 in the same SAHF-positive cells were detected by immunofluorescent staining (Fig. 4, A and B). To study the roles of endogenous p16 or ATF4, we transfected RTECs with p16-siRNA or ATF4-siRNA. The transfection efficiency of ATF4 siRNA was detected to be as high as 85.67 ± 4.23%, and the expression of ATF4 mRNA and protein was dramatically inhibited by ATF4 siRNA (data not shown). Interestingly, suppressing endogenous p16 or ATF4 prevented ER stress-induced premature senescence in PTECs, as indicated by reduced SA-β-gal-, SAHF-positive cells and the fraction of cells in G0/G1 phases (P < 0.05, Fig. 4, C–H).

AGE-induced premature senescence of RTECs depends on activation of ATF4/p16 signaling. Similar to AGE expression, little ATF4 deposition was detected in the normal control group, while dramatically increased ATF4 deposition was observed in RTECs from the DN patients (Fig. 5A). Linear correlation analyses show that nuclear ATF4 expression was positively correlated with AGE deposition (P < 0.05, Fig. 5B).

In vitro studies also show that upregulation of ATF4 induced by AGEs was also dose and time dependent (data not shown). The expression of ATF4 and p16 was colocalized in the nucleus of the same SAHF-positive cells (Fig. 5, C and D). ATF4 siRNA resulted in a significant decrease in AGE-induced p16 expression and premature senescence of PTECs (P < 0.05, Fig. 5, E–I).
Fig. 5. AGEs induce premature senescence of RTECs by ER stress-dependent ATF4/p16 signaling. A: immunohistochemistry detected nuclear ATF4 expression in RTECs (magnification ×200). B: linear correlation analyses show that nuclear ATF4 expression was positively correlated with AGE deposition. **P < 0.05. C, D: RTECs were treated with 7.5 µM AGE-BSA or BSA for 48 h. Immunofluorescence shows that ATF4 and p16 were colocalized in the nucleus of the same SAHF-positive cells after AGE stimulation. E, F: RTECs were transfected with control siRNA (50 nM) or ATF4 siRNA (50 nM) for 24 h and then cultured with 7.5 µM AGE-BSA or BSA for 48 h. Western blot showed ATF4 siRNA transfection significantly reduced AGEs-induced p16 expression. G–I: RTECs were transfected with control siRNA (50 nM) or ATF4 siRNA (50 nM) for 24 h and then cultured with 7.5 µM AGE-BSA or BSA for 48 h. The SA-β-gal, SAHF-positive cells and cells in the G0/G1 phase were significantly reduced in the ATF4 siRNA-transfected group compared with those in the AGE group. **P < 0.05. Results are representative from experiments performed in triplicate.
In this study, we demonstrate that AGEs induced ER stress-regulated ATF4 activation and p16 upregulation, which play critical roles in the premature senescence of RTECs during DN progression (Fig. 6).

It has been demonstrated that premature senescence, as a programmed biological process, is involved in the initiation and development of several chronic diseases (7, 26, 36). Premature senescence was observed in renal cells under conditions of high glucose, oxidative stress, or ischemia reperfusion injury (17, 18, 35). p21 and p16 pathways are very important signaling pathways of premature senescence in renal inherent cells (23). We recently showed that p21 siRNA partially inhibits AGE-induced premature senescence (22). The cell cycle regulator p16 is strongly associated with aging in the kidneys of humans and rodents (19, 24). p16 expression in RTECs was positively correlated with proteinuria and glycosylated hemoglobin (HbA1c) level (4). Our in vivo study found that p16 expression is extensively upregulated in RTECs accompanied by increased deposition of AGEs. In vitro studies further demonstrate that AGEs time- and dose-dependently induced p16 activation, whereas deletion of endogenous p16 with siRNA decreased AGE-induced premature senescence in RTECs, suggesting that p16 is a critical modulator in premature senescence during DN.

Emerging evidence has illustrated the importance of ER stress through coupling oxidative stress, regulation of inflammatory responses, and autophagy in metabolic and age-related diseases (14, 30). The appropriate balance between ER stress and advanced glycation may be important for cellular homeostasis during aging (16, 28). A recent study has reported that ER stress directly accelerated oncogene-induced premature senescence in human melanocytes through a p16-independent pathway (8). Contrary to their result, we found that ER stress marker GRP78 is colocalized with p16 in the same RTECs during DN, indicating that ER stress may be involved in p16-mediated premature senescence. In our vitro studies further demonstrate that AGEs greatly upregulated the expression of GRP78. In addition, GRP78 and p16 were both expressed in SAHF-positive cells. We further found that inhibition of the ER stress by 4-PBA effectively abolished AGE-induced p16 expression and premature senescence. Taken together, these data confirm that ER stress mediated AGE-induced premature senescence through the p16 signaling pathway.

Transcription factor ATF4 is a major signaling molecule of ER stress. After activation, ATF4 translocates from the cytoplasm to the nucleus to modulate downstream gene expression (2). Although studies have found that ATF4 expression in kidney tissues is not obviously altered with aging (15), the expression of ATF4 is involved in cell cycle by regulation of the gene expression of p21, p27, and p16 in the progression of age-related diseases (3, 10, 11, 13, 21). A recent study showed that ATF4 plays a critical role in retinal inflammatory signaling and Muller cell-derived inflammatory cytokine production in diabetes (38). Here, our results show that GRP78 and ATF4 or p16 were colocalized in the same SAHF-positive cells exposed to ER stress inducers, and suppressing endogenous ATF4 or p16 expression successfully prevented premature senescence induced by ER stress inducers, suggesting that the ATF4/p16 pathway is implicated in ER stress-mediated premature senescence. We further found that ATF4 and p16 were colocalized in the same senescent RTECs during DN as well as AGE-treated RTECs. ATF4 gene silence significantly reduced p16 gene expression and premature senescence induced by AGEs. Therefore, we speculate that ER stress-regulated ATF4/p16 signaling activation is critical in AGE-induced premature senescence. Based on the data that ATF4 overexpression directly induced p16 activation and premature senescence, which successfully attenuated by p16 gene silencing, our results clearly demonstrate that activation of the ATF4/p16 signaling, secondary to ER stress, is a key factor in AGES-induced premature senescence (Fig. 6).

In conclusion, our findings show that ER stress-regulated ATF4/p16 signaling accelerates premature senescence in RTECs and is associated with DN progression. These data may provide a potential novel therapeutic target for the prevention of DN progression.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES
ATF4/p16 SIGNALING AND PREMATURE SENESCENCE OF DN


