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Sublytic C5b-9 triggers glomerular mesangial cell apoptosis in rat Thy-1 nephritis via Gadd45 activation mediated by Egr-1 and p300-dependent ATF3 acetylation

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The apoptosis of glomerular mesangial cells (GMCs) is considered to be an important contributor to the initiation and development of rat Thy-1 nephritis (Thy-1N) and is accompanied by sublytic C5b-9 deposition. However, the mechanism by which sublytic C5b-9 triggers GMC apoptosis has not been elucidated. In this study, functional and histological examinations were performed on GMCs treated with sublytic C5b-9 (in vitro) and renal tissues of Thy-1N rats (in vivo). The in vitro studies found that sublytic C5b-9 could trigger GMC apoptosis through upregulating Egr-1, ATF3, and Gadd45 expression. Egr-1-mediated post-transcriptional modulation of ATF3, Egr-1/ATF3-enhanced Gadd45 promoter activity, and p300-mediated ATF3 acetylation were all involved in GMC apoptosis. More importantly, the effective binding elements for Egr-1 and ATF3 to Gadd45/β promoters and the ATF3 acetylation site were identified. In vivo, silencing renal p300, Egr-1, ATF3, and Gadd45/β/γ significantly decreased GMC apoptosis, secondary GMC proliferation, and urinary protein secretion in Thy-1N rats. Together, these findings implicate that sublytic C5b-9-induced activation of Egr-1/p300–ATF3/Gadd45 axis plays a critical role in GMC apoptosis in Thy-1N rats.

Keywords: glomerular mesangial cells, sublytic C5b-9, Thy-1 nephritis, transcription factor, acetylation, apoptosis

Introduction

Mesangiproliferative glomerulonephritis (MsPGN) is a disease with high incidence in humans (Tumlin et al., 2007; Tamouza et al., 2012). Several studies have demonstrated that complement, especially C5b-9, leads to GMC damage (Onda et al., 2011; Pickering and Cook, 2011). However, the mechanism by which C5b-9 causes GMC lesions has not been fully elucidated. Rat Thy-1 nephritis (Thy-1N) is a well-known model of MsPGN (Cove-Smith et al., 2013), and multiple evidences have suggested that C5b-9 is the main mediator of GMC lesion in Thy-1N rats (Liu et al., 2012; Cantaluppi et al., 2015). Previous studies have proved that nucleated cell injury mediated by C5b-9 is almost non-lytic (sublytic) (Kitzler et al., 2012; Du et al., 2014). Sublytic C5b-9 can trigger diverse responses, including apoptosis, proliferation, and cytokine release (Qiu et al., 2011; Triantafillou et al., 2013; Zhang et al., 2014a). Increasing evidences support that GMC apoptosis is an important contributor to the initiation and development of Thy-1N (Amore and Coppo, 2000; Qiu et al., 2014). Nevertheless, the mechanism governing GMC apoptosis by sublytic C5b-9 remains unclear.

It is well established that cell apoptosis is associated with the activation of certain transcription factors upon stimulations (Magro and Wang, 2013). Early growth response gene 1 (Egr-1), a transcription factor, has been shown to play roles in multiple pathways for differentiation, proliferation, and apoptosis (Shin et al., 2010; Zwang et al., 2012). Early studies have revealed that Egr-1 was closely correlated with serum-induced proliferation of rat GMCs (Rupprecht et al., 2000), and might play an role in renal fibrosis via interacting with nuclear factor of activated T cells and Sp1/Sp3 to regulate membrane type 1 matrix metalloproteinase in GMCs (Alfonso-Jaume et al., 2004). However, the role of Egr-1 in GMC apoptosis has not been reported. Analogously, activating transcription factor 3 (ATF3), a member of ATF/cyclic AMP response element-binding (CREB) family, can also be induced by various stress stimuli and has...
dual effects on cell fate, i.e. apoptosis or proliferation (Thompson et al., 2009; Pu et al., 2015). Reportedly, Egr-1 can transcriptionally enhance ATF3 expression and trigger apoptosis in HCT116 (Cho et al., 2007), and ATF3 can upregulate (Kwon et al., 2012) or downregulate (Park et al., 2013) Egr-1 expression in colon cancer cells. Our previous microarray data have revealed that Egr-1 and ATF3 are two early co-upregulated genes in both renal tissues of Thy-1N rats and cultured GCMs attacked by sublytic C5b-9. Thus, the relationship between Egr-1 and ATF3 and their roles in sublytic C5b-9-induced GMC apoptosis should be further studied.

Furthermore, we have proved that growth arrest- and DNA damage-inducible protein 45 (Gadd45) α (Jiang et al., 2010) and Gadd45γ (Xu et al., 2006) are involved in GMC apoptosis, and ATF3 can promote GMC apoptosis via Gadd45α upregulation (Xu et al., 2011). Besides, Egr-1 can regulate Gadd45α/β expression upon ultraviolet radiation b (UVB) irradiation (Thyss et al., 2009). To further explore the mechanism underlying GMC apoptosis in response to sublytic C5b-9, the regulation among Egr-1, ATF3, and Gadd45 needs to be clarified.

EIA-binding protein p300 (p300), a transcriptional co-activator with acetyltransferase activity for transcription factors and histones, can regulate the expression of target genes (Bedford and Brindle, 2012). It has been demonstrated that Egr-1 can be acetylated by p300 in serum-stimulated prostate cancer cells (Yu et al., 2004), and p300 can also interact with ATF4 (Lassot et al., 2005) and ATF5 (Liu et al., 2011). Given that our previous microarray data showed upregulation of p300, whether p300 can acetylate Egr-1 and ATF3 and subsequently alter their functions needs further study.

In this study, the elevated Egr-1 induced by sublytic C5b-9 can promote ATF3 mRNA stability to maintain ATF3 protein abundance, and both Egr-1 and ATF3 can facilitate Gadd45 gene transcription. Meantime, upregulated p300 can bind to and acetylate ATF3 at lysine 42, which further enhances the transcriptional activity of Gadd45 genes. The activation of Egr-1/p300–ATF3/Gadd45 axis in GMCs triggered by sublytic C5b-9 serves a permissive role in GMC apoptosis in Thy-1N rats.

Results

Upregulation of Egr-1, ATF3, and Gadd45 in GMC apoptosis induced by sublytic C5b-9

Our previous studies found that the expression of ATF3, Gadd45α, and Gadd45γ was elevated in GCMs attacked by sublytic C5b-9 (Qiu et al., 2009; Xu et al., 2011). Present experiments showed that the expression of Egr-1, ATF3, and Gadd45α/β/γ increased in a time-dependent manner in renal tissues of Thy-1N rats, with mRNA levels peaked at 1 h uniformly and protein levels peaked at 2 and 3 h, respectively (Figure 1A, B and Supplementary Figure S1A). Additionally, GMC apoptotic changes, i.e. irregular chromatin aggregation at the nucleus periphery and chromatin condensation, were observed in Thy-1N rats at 3 h (Supplementary Figure S1B).

In vitro, the formation of C5b-9 was found in cultured rat GCMs (Supplementary Figure S1C), and an increase in the expression of Egr-1, ATF3, and Gadd45α/β/γ was observed (Figure 1C and Supplementary Figure S1D). When GCMs were treated with modified Eagle’s medium (MEM), Thy-1 Ab, Thy-1 Ab + HIS, Thy-1 Ab + C6DS, or Thy-1 Ab + C6DS + C6 for 1 h or 3 h, only Thy-1 Ab + C6DS + C6 (adding recombination C6 back to C6DS) remarkably elevated the expression of these proteins as well as sublytic C5b-9 treatment (Figure 1D and Supplementary Figure S1E). Meantime, GMC apoptosis was significantly increased in sublytic C5b-9 and Thy-1 Ab + C6DS + C6 groups (Supplementary Figure S1F). These results suggest that upregulation of Egr-1, ATF3, and Gadd45α/β/γ and GMC apoptosis could be due to sublytic C5b-9 assembly on GMC membranes.

Subsequently, overexpression of Egr-1, ATF3, and Gadd45α/β/γ markedly increased GMC apoptosis, while knockdown of them obviously reduced GMC apoptosis (Figure 2 and Supplementary Figure S2A–F), indicating that Egr-1, ATF3, and Gadd45α/β/γ play important roles in sublytic C5b-9-induced GMC apoptosis.

Egr-1 post-transcriptionally regulates ATF3 abundance by stabilizing ATF3 mRNA in GMCs stimulated with sublytic C5b-9

Given that Egr-1 was upregulated earlier than ATF3, we supposed that Egr-1 might regulate ATF3 expression. As shown in Figure 3A, Egr-1 overexpression in GMCs markedly increased ATF3 protein abundance, while Egr-1 knockdown significantly inhibited ATF3 protein level upon sublytic C5b-9 stimulation. However, ATF3 mRNA level did not change upon Egr-1 overexpression or knockdown (Figure 3B), suggesting that Egr-1 may regulate ATF3 expression at a post-transcriptional level. Conversely, ATF3 overexpression or knockdown did not manifest significant changes in the Egr-1 protein level in GMCs (Supplementary Figure S3A), indicating ATF3 downstream to Egr-1 in GMCs. Next, proteasome inhibitor MG132 treatment increased the total level of ubiquitination in GMCs and restored ATF3 upregulation in both shCTR and shEgr-1 groups, while the predominant form of ubiquitinated ATF3 was diminished in shEgr-1 group (Figure 3C and Supplementary Figure S3B), suggesting that Egr-1 regulates ATF3 protein expression independently of ubiquitin proteasome pathway. Rather, Egr-1 interference markedly destabilized ATF3 mRNA as shown by the decay of ATF3 mRNA (Figure 3D), implicating that Egr-1 probably upregulates ATF3 protein abundance via enhancing ATF3 mRNA stability.

Then, we further excluded the possibility that Egr-1 regulates ATF3 at a transcriptional level. Since the rat ATF3 promoter nucleotide sequences have not been sequenced yet (Supplementary Figure S3C), genome walking was used to identify the flanking unknown nucleotide sequences in front of the given DNA region in ATF3 gene (Supplementary Figure S3Cii). After three nested polymerase chain reaction (PCR; Supplementary Figure S3Ciii), a 430-bp nucleotide sequence of ATF3 proximal promoter was obtained (GenBank KT210895), and Basic Local Alignment Search Tool showed 97% similarity between rat and mouse ATF3 (Supplementary Figure S3D). In addition, TFsearch software could not predict any Egr-1-binding site on mouse or rat ATF3 proximal promoter (data not shown). Correspondingly, Egr-1 did not affect ATF3 promoter activity (Supplementary Figure S3E). Thus, we
confirmed that Egr-1 post-transcriptionally regulates ATF3 expression. Notably, consistent with ATF3 being Egr-1 downstream, ATF3 knockdown abrogated the proapoptotic effect of Egr-1, while ATF3 overexpression enhanced GMC apoptosis mitigated by shEgr-1 (Figure 3E).

**Egr-1 and ATF3 trigger Gadd45α/β/γ gene transcription in GMCs stimulated by sublytic C5b-9**

As exhibited in Figure 4A–C, overexpression or knockdown of Egr-1 and ATF3 increased or decreased the expression and promoter activity of Gadd45 genes, respectively, suggesting that sublytic C5b-9-induced Egr-1 and ATF3 function as positive regulators of Gadd45 gene transcription.

Given that we have demonstrated Gadd45α is the target gene of ATF3 (Xu et al., 2011), here, we focus on the roles of Egr-1 and ATF3 in regulating Gadd45β/γ gene transcription. Several Egr-1-binding element (EE) sites and ATF3-binding element (AE) sites on the Gadd45β/γ promoters have been predicted using TFsearch software (Figure 4D and E, upper). For Gadd45β promoter, truncated promoter fragments (−146 to +236 nt and
+23 to +236 nt) co-transfected with pRES2/Egr-1 and the shortest promoter fragment (+23 to +236 nt) co-transfected with pRES2/ATF3 displayed reduced Gadd45β promoter activity (Figure 4D, lower), indicating that EE1 (−227 to −212 nt) and EE2 (−86 to −74 nt) might be the effective Egr-1-binding sites, and AE3 (+2 to +13 nt) might be an effective ATF3-binding element. For Gadd45γ promoter, fragments (−211 to +72 nt and −61 to +72 nt) with Egr-1 or ATF3 overexpression both
exhibited acute reduction in luciferase activity (Figure 4E, lower), implying that Gadd45γ promoter region (−456 to −61 nt) might contain >1 effective Egr-1 and ATF3-binding elements, including EE (−182 to −170 nt), AE2 (−213 to −203 nt), and AE3 (−188 to −177 nt). Although no putative Egr-1 and ATF3-binding sites in the region (−456 to −211 nt) were predicted by the computer program, it cannot be excluded that there might be EEs and AEs.

To further determine the binding sites on Gadd45β/γ proximal promoters for Egr-1 and ATF3, we performed chromatin immunoprecipitation (ChIP) assay. As shown in Figure 4F, GMCs with sublytic C5b-9 attack could induce endogenous Egr-1 binding to the sequences (−139 to +115 nt and −320 to −110 nt) of Gadd45β promoter (which might be EE1 and EE2) and ATF3 binding to the sequence (−139 to +115 nt) of Gadd45β promoter (AE3 probably). Unexpectedly, we observed faintly faint binding between ATF3 and the sequence (−320 to −110 nt), suggesting that ATF3 may bind to this region by interacting with other transcription factors. For Gadd45γ promoter, sublytic C5b-9 could increase Egr-1 and ATF3 binding to the sequences (−198 to +28 nt and −467 to −198 nt), which further supported that there were effective Egr-1 and ATF3-binding sites within the region (−456 to −211 nt) of Gadd45γ promoter. Subsequently, we identified that Egr-1 could not bind to ATF3 for their

Figure 3 Effects of Egr-1 on ATF3 expression and GMC apoptosis induced by sublytic C5b-9. (A and B) GMCs were transfected with pIRES2, pIRES2/Egr-1, shCTR or shEgr-1 for 48 h, and then stimulated with (or without) sublytic C5b-9 for 1 h. Protein and mRNA were determined using IB (A) and quantitative real-time PCR (qPCR) (B), respectively. n.s., no significant difference. (C) GMCs were transfected with shCTR or shEgr-1 for 48 h, and then exposed to sublytic C5b-9 with (or without) MG132 (10 μM) for 3 h. Then, total cell lysate was immunoprecipitated using anti-ATF3 Ab, and the ubiquitination level of ATF3 was analyzed by IB. (D) GMCs transfected with shCTR or shEgr-1 were treated with sublytic C5b-9 for 1 h, followed by 5 μM actinomycin D exposure in the presence of 4% NHS for the indicated time. Then, ATF3 mRNA level was measured using qPCR. *P < 0.05, **P < 0.01 vs. shCTR group at each indicated time. (E) GMCs co-transfected with the indicated plasmids were challenged with (or without) sublytic C5b-9 for 3 h, and then flow cytometry was used to measure the number of apoptotic GMCs. #P < 0.01 vs. pIRES2/Egr-1 + shCTR group; ∆#P < 0.01 vs. shEgr-1 + pIRES2 + sublytic C5b-9 group.

Sublytic C5b-9 triggers apoptosis via Egr-1/p300–ATF3/Gadd45

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Figure 4 Roles of Egr-1 and ATF3 in Gadd45α/β/γ activation in GMCs upon sublytic C5b-9 stimulation. (A and B) GMCs transfected with various plasmids were stimulated with (or without) sublytic C5b-9. Gadd45α (2 h) and Gadd45β/γ (1 h) mRNA levels Gadd45α/β/γ (3 h) protein level were determined by qPCR (A) and IB (B), respectively. (C) GMCs were transfected with Gadd45α/β/γ full-length promoters accompanied with various vectors for 48 h, followed with (or without) sublytic C5b-9 attack for 3 h. Then, luciferase reporter analysis was performed. *P < 0.05, **P < 0.01 vs. MEM and pIRES2 groups; **P < 0.01 vs. shCTR + sublytic C5b-9 and sublytic C5b-9 groups. (D) Schematic representation of Gadd45β promoter deletion mutants (upper). GMCs were transfected with pGL3-Gadd45β-FL or different promoter deletion fragments accompanied with pIRES2/Egr-1 or pIRES2/ATF3 for 48 h, followed with luciferase reporter assays (lower). **P < 0.01 vs. pGL3-Gadd45β-FL group; **P < 0.01 vs. pGL3-Gadd45β (−561 to +236 nt), pGL3-Gadd45β (−319 to +236 nt), and pGL3-Gadd45β (−146 to
transcription function (Supplementary Figure S3F). These findings collectively reveal that Egr-1 and ATF3 can directly trigger Gadd45β/γ gene transcription. Together, our data suggest that sublytic C5b-9 can induce GMC apoptosis through Egr-1–ATF3–Gadd45 axis activation.

**ATF3 rather than Egr-1 is acetylated via p300 at lysine 42 in GMCs stimulated by sublytic C5b-9**

Our previous experiments showed that p300 was co-upregulated in vivo and in vitro (Qiu et al., 2014). Here, we also demonstrate that p300 expression was increased in vitro, with both mRNA and protein levels peaked at 3 h (Supplementary Figure S4A and B), and sublytic C5b-9 as well as Thy-1 Ab + C6DS + C6 significantly enhanced p300 level in GMCs than other treatments (Supplementary Figure S4C, D), further confirming that p300 elevation was really induced by sublytic C5b-9.

Next, immunoprecipitation (IP) analysis showed an interaction between p300 and ATF3 but not Egr-1, which reached the maximum at 3 h after sublytic C5b-9 stimulation (Figure 5A). Meanwhile, acetylation level of ATF3 was increased, peaked at 3 h, and returned to baseline by 12 h (Figure 5B), but Egr-1 acetylation was not detected (Supplementary Figure S4E).

Additionally, only GMCs upon sublytic C5b-9 or Thy-1 Ab + C6DS + C6 significantly enhanced p300 level in GMCs than other treatments (Supplementary Figure S4A, B, and Supplementary Figure S4C, D), further confirming that p300 elevation was really induced by sublytic C5b-9.

ATF3 acetylation mediated by p300 is required for sublytic C5b-9–induced Gadd45β/γ expression and GMC apoptosis

To investigate the roles of p300-dependent ATF3 acetylation in regulating Gadd45β/γ expression and GMC apoptosis, GMCs were transfected with shp300, ATF3 (WT), or ATF3 (K42R). The data showed that shp300 and ATF3 (K42R) not only reduced Gadd45β/γ expression (Figure 6A and Supplementary Figure S5A) and promoter activity (Figure 6B), but also inhibited ATF3 binding to Gadd45β/γ gene promoters (Figure 6C) and GMC apoptosis (Figure 6D). Moreover, shp300 and ATF3 (K42R) also decreased Gadd45α expression (Figure 6A), suggesting that ATF3 acetylation can affect the expression of all three members of Gadd45 family. Furthermore, p300 interference could not prevent the nuclear transport of ATF3 (Supplementary Figure S5B), implying that ATF3 acetylation by p300 does not affect the subcellular localization, but affect the transcriptional activity of ATF3.

Since we observed that AE3 on Gadd45β promoter and AE3 on Gadd45γ promoter had an overlapping p300-binding element (Figure 4D and E, upper), further studies were performed to determine whether p300 could bind to Gadd45β/γ promoters directly or together with ATF3 as a complex. Re-ChIP assay showed that shATF3 could eliminate p300 binding to the Gadd45β/γ promoters (Figure 6E), revealing that it was ATF3-dependent. Subsequently, chromatin immunodepletion (ChID) exhibited that depletion of acetylated protein removed all ATF3-bound promoter regions, while Re-ChIP assay manifested that re-IP of acetylated ATF3 restored ATF3-bound promoter regions (Figure 6F), suggesting that ATF3 acetylation by p300 is essential for ATF3/p300 complex binding to AEs. Collectively, these findings reveal that ATF3 and p300 bind to the AEs by forming a complex, which subsequently enhances Gadd45β/γ transcription and GMC apoptosis.

**Silence of renal p300, Egr-1, ATF3, or Gadd45β/γ suppresses GMC apoptosis, pathological changes, and urinary protein secretion in Thy-1N rats**

The in vivo experiments showed that, consistent with the in vitro data, the level of ATF3 acetylation as well as the interaction between p300 and ATF3 increased in Thy-1N rats, while Egr-1 could not co-precipitate with p300 (Figure 7A and B). In addition, Thy-1N rats pretreated with LV-shp300, LV-shEgr-1, or LV-shATF3 via renal perfusion exhibited reduced expression of p300, Egr-1, ATF3, and Gadd45β/γ in renal tissues (Figure 7C and Supplementary Figure S6A, B). However, pretreatment with LV-shEgr-1 also decreased ATF3 expression (Figure 7C and Supplementary Figure S6B).
Figure 5 p300 mediates ATF3 acetylation at lysine 42 in GMCs with sublytic C5b-9 stimulation. (A and B) GMCs were treated with sublytic C5b-9 for various time points, and cell lysates were subjected to IP using antibodies against p300 (A) or ATF3 (B). Contents of Egr-1, ATF3, p300, and acetylated lysine (Ac-K) in the complexes were measured by IB assay. *P < 0.05, **P < 0.01 vs. 0 h (non-treated). (C) GMCs were exposed to different treatments as indicated for 3 h. IP samples by anti-ATF3 Ab were analyzed by IB using antibodies against Ac-K, ATF3,
more important, knockdown of renal p300 by LV-shp300 inhibited ATF3 acetylation after Thy-1N induction (Figure 7D).

Furthermore, TUNEL staining and electron microscopy (EM) showed remarkably decreased TUNEL-positive cell numbers and GMC apoptotic changes at 3 h in Thy-1N rats pretreated with LV-p300, LV-Egr-1, LV-ATF3, or LV-Gadd45β/γ (Figure 8A and B, Supplementary Figure S6D). Moreover, GMC proliferation and ECM accumulation in Thy-1N rats pretreated with LV-shp300, LV-shEgr-1, LV-shATF3, or LV-Gadd45β/γ were decreased on day 7 (Figure 8C and D). Finally, all these pretreated Thy-1N rats showed a reduction in the urinary protein content on day 7 (Figure 8E). Taken together, these data suggest that silence of renal p300, Egr-1, ATF3, or Gadd45β/γ can decrease GMC apoptosis, mitigate secondary GMC proliferation, and ameliorate the renal function of Thy-1N rats.

Discussion

In this study, we confirmed that Egr-13, ATF3, and Gadd45α/β/γ expression was co-upregulated both in vivo and in vitro, which could promote GMC apoptosis triggered by sublytic Cs5b-9. Reportedly, Egr-1 could regulate ATF3 expression, and ATF3 could also modulate Egr-1 level under different conditions (Cho et al., 2007; Kwon et al., 2012; Park et al., 2013). Here, our experiments manifested that Egr-1 could upregulate ATF3 protein by enhancing ATF3 mRNA stability rather than an ubiquitination and proteasomal pathway. Previous studies showing that MG132 activates c-Jun N-terminal kinase (JNK) to initiate apoptosis (Pietkiewicz et al., 2013) and ATF3 is a downstream molecule of JNK (Pu et al., 2015) may explain why MG132 restored ATF3 protein while not influencing ATF3 ubiquitination. The mechanism underlying Egr-1-mediated ATF3 mRNA stabilizing remains unclear. A few RNA-binding proteins bind to 3' untranslated regions with AU-rich elements and stabilize gene transcripts (Kundu et al., 2012; Lu et al., 2015). It was also reported that HuR could inhibit ATF3 expression by retarding translocation of ATF3 transcript to the cytosol (Choi et al., 2009). Whether Egr-1 can modulate the behavior of RNA-binding proteins like HuR and thus stabilize ATF3 mRNA should be further studied. Our results also demonstrate the proapoptotic effect of Egr-1 in GMCs upon sublytic Cs5b-9 attack, which was mediated by ATF3 downstream of Egr-1.

In this study, we identified the rat ATF3 proximal promoter sequences by genome walking (Fang et al., 2013), which further proved that the regulation of Egr-1 to ATF3 was not at a transcriptional level but post-transcriptionally by promoting ATF3 mRNA stability.

Egr-1 has been reported to induce cell apoptosis by enhancing p53 (Zhang et al., 2012) or PTEN (Viroille et al., 2001) expression, and also can promote survival via p21 induction (Kim et al., 2014). Similarly, ATF3 alone, or in combination with other stress-responsive proteins like c-Jun, can bind to ATF/CREB consensus sequence (Hai and Curran, 1991) and mediate apoptosis (Pu et al., 2015) or proliferation (Zhou et al., 2014).

Our results displayed that both Egr-1 and ATF3 increase the expression and promoter activity of Gadd45α/β/γ in GMCs stimulated by sublytic Cs5b-9, implying that sublytic Cs5b-9 may upregulate Gadd45α/β/γ via Egr-1 and ATF3-mediated transcriptional activation.

Previously, we found that ATF3 could elevate Gadd45α transcription by binding to the region (−133 to −122 nt) of Gadd45α promoter (Xu et al., 2011). Here, we identified several Egr-1-binding sites and ATF3-binding sites in the −23 nt region of Gadd45β promoter and −456 to −61 nt region of Gadd45γ promoter. Interestingly, the Gadd45β promoter fragment (−561 to +236 nt) co-transfected with pIRE2-Egr-1 and fragments (−561 to +236 nt and −319 to +236 nt) co-transfected with pIRE2/ATF3 exhibited higher Gadd45β promoter activity (Figure 4D), implying putative negative control elements in the region (−1105 to −319 nt) of Gadd45β promoter. Furthermore, ChIP assays revealed, for the first time, that Egr-1 and ATF3 could specifically bind to the Gadd45β promoter fragments (−139 to +115 nt and −320 to −110 nt, including EE1, EE2, and AE3) and Gadd45γ promoter fragments (−198 to +28 nt and −467 to −198 nt, including EE, AE2, and AE3).

It has been reported that Egr-1 undergoes acetylation by p300 in response to growth stimuli (Yu et al., 2004). Our results revealed, for the first time, that ATF3 rather than Egr-1, could be acetylated at lysine 42 upon sublytic Cs5b-9 attack in GMCs. p300 was shown to interact with ATF4 (Lassot et al., 2005) and ATF5 (Liu et al., 2011), both belonging to the ATF/CREB family. Here, p300 and ATF3 form a complex to bind to Gadd45β/γ promoters through ATF3 binding to its elements. Notably, only acetylated ATF3 could bind to Gadd45β/γ promoters. These findings indicate that ATF3 acetylation by p300 is essential for ATF3 binding to Gadd45β/γ promoters and subsequently promotes Gadd45β/γ transcription and GMC apoptosis induced by sublytic Cs5b-9.

Our in vivo experiments indicate that Egr-1/p300–ATF3/Gadd45 axis is necessary for Thy-1N development. It is worth mentioning that GMC apoptosis triggered by sublytic Cs5b-9 was also found to be associated with the expression of other genes, i.e. interferon regulatory factor-1 and X-linked inhibitor of
apoptosis-associated factor-1 (Qiu et al., 2014), suggesting a synergetic effect of many apoptosis-related genes during Thy-1N. Since sublytic C5b-9 and other complement components are detected in the glomeruli and urine of patients with MsPGN (Onda et al., 2011; Pickering and Cook, 2011), such complement system-related, transcription factors-mediated apoptosis pathways may be also involved in MsPGN progression.

In summary, our study reveals that sublytic C5b-9 leads to the upregulation of Egr-1 and ATF3, which bind to Gadd45 promoters (−133 to −122 nt of Gadd45α, −139 to +115 nt and
−320 to −110 nt of Gadd45β, −198 to +28 nt and −467 to −198 nt of Gadd45γ), facilitating Gadd45 gene transcription and subsequent GMC apoptosis. During this process, Egr-1 post-transcriptionally modulates ATF3 abundance by stabilizing ATF3 mRNA. Meantime, elevated p300 can bind to and acetylate ATF3 at lysine 42, which enhances the transcriptional activity of ATF3 on Gadd45 genes and finally induces GMC apoptosis (Figure 8F). Collectively, these findings add a new piece of important information to understanding the mechanism of GMC apoptosis mediated by sublytic C5b-9 in rat Thy-1N, which may provide a novel insight to the pathogenesis of human MsPGN.

**Materials and methods**

*Animals, cell line, and reagents*

Male Sprague-Dawley (SD) rats (180–200 g) were purchased from B&K Universal Ltd. All animal experiments were performed according to the animal care and use committee guidelines. Rat GMC strain (HBZY-1) was provided by China Centre for Type Culture Collection. Normal human serum (NHS) from several healthy adult donors was used as a source of serum complement, and heat-inactivated serum (HIS) was obtained by incubating NHS at 56°C for 30 min. Human complement C6-deficient serum (C6DS) was obtained from Complement Technology Inc. Recombinant
human C6 was purchased from Sino Biological Inc. Rabbit polyclonal anti-Thy-1 serum (Thy-1 antibody, Thy-1 Ab) was prepared according to previously published procedures (Wang et al., 2006). Rabbit normal serum (NS) was prepared from New Zealand rabbit.

Antibodies and other reagents are listed in Supplementary Materials and methods.

**Figure 8** Effects of p300, Egr-1, ATF3, and Gadd45β/γ knockdown on GMC apoptosis, pathological changes, and urinary protein secretion in Thy-1N rats. (A) The numbers of apoptotic cell at 3 h after Thy-1N induction were counted by fluorescence microscopy with TUNEL staining. (B) Ultrastructural changes at 3 h including the irregular aggregation of chromatin in the periphery of nucleus and nuclear chromatins condensation were detected by EM. (C) Changes in glomerular cells on day 7 were measured by H&E staining under light microscopy (LM). (D) Ultrastructural changes on day 7 were examined by EM. (E) Total contents of urinary protein (mg per 24 h) on day 7 were detected. **P < 0.01 vs. shCTR + Thy-1N and Thy-1N groups. (F) A putative scheme for the molecular regulation of GMC apoptosis triggered by sublytic C5b-9. In response to sublytic C5b-9, Egr-1, ATF3, p300, and Gadd45 are all upregulated. Egr-1 post-transcriptionally upregulates ATF3 abundance by stabilizing ATF3 mRNA. The elevated p300 can bind to and acetylate ATF3 at lysine 42. Finally, Egr-1 and p300–ATF3 complex bind to the promoters of Gadd45 to enhance Gadd45 transcription and promote GMC apoptosis. Scale bar, 20 μm (A), 5 μm (B, D), and 50 μm (C).
Gadd45β, and Gadd45γ were constructed. See Supplementary Materials and methods for details.

**GMC culture and sublytic C5b-9 determination**

Culture of rat GMCs and determination of sublytic C5b-9 were previously described (Qiu et al., 2009). Of note, 5% Thy-1 Ab and 4% NHS were used in our study, with <0.5% lactate dehydrogenase release regarded as a sublytic effect. GMCs were also treated with 5% Thy-1 Ab, 5% Thy-1 Ab + 4% H1S, 5% Thy-1 Ab + 4% C6DS, 5% Thy-1 Ab + 4% C6DS + C6 (2 mg/L), or MEM (Qiu et al., 2014).

**GMC transfection, retrovirus infection, and identification**

GMCs were transfected with corresponding plasmids by using Neon transfection system (Invitrogen) as previously described (Qiu et al., 2014). The transfection efficiency was examined by the fluorescence of green fluorescence protein (GFP) or expression of corresponding proteins. For retrovirus infection, 0.5 × 10^6 GMCs in 96-well cell culture cluster were cultured with lentivirus at the titer of 5 × 10^6 or 1 × 10^7 TU/ml for 60 h to determine whether target cells could be infected by the lentivirus efficiently.

**Thy-1N model establishment and experimental design**

SD rats were divided into two groups (n = 9 in each time point/group), namely (i) Thy-1N group: rats were given Thy-1 Ab (0.75 ml/100 g) by an intravenous injection. (ii) NS group: rats were injected with rabbit NS (0.75 ml/100 g). Renal cortex samples were obtained at fixed time points.

To confirm the roles of p300, Egr-1, ATF3, Gadd45β, and Gadd45γ in apoptotic and proliferative changes of Thy-1N rats, SD rats were divided into eight groups (n = 8–10 in each time point/group), namely (i) Thy-1N, (ii) Thy-1N, (iii) Lv-shp300 + Thy-1N, (iv) Lv-shEgr-1 + Thy-1N, (v) Lv-shATF3 + Thy-1N, (vi) Lv-shGadd45β + Thy-1N, (vii) Lv-shGadd45γ + Thy-1N, and (viii) Lv-shCTR + Thy-1N. First, LV-shRNAs were infused into rat kidney via renal artery perfusion followed immediately by renal vein occlusion for 10 min (Zhang et al., 2014a). Then, on day 4 after perfusion, rats were treated with Thy-1 Ab to induce Thy-1N. Renal cortex samples were collected at 2 and 3 h and on day 7. GFP was observed on 96-h frozen sections of renal tissues, defining the efficiency of transferring LV-shRNA into kidneys, and the effect of corresponding gene silencing was evaluated by IB assay. Additionally, pathological changes of kidneys were observed by LM and EM.

**IB and immunoprecipitation**

For IB, GMCs and renal tissues were lysed and quantified. Whole-cell lysates were electrophoresed and transferred onto polyvinylidene difluoride membrane. The blots were blocked, probed with indicated primary antibodies and incubated with respective DyLight-conjugated secondary antibodies. Finally, the blots were detected using a two-color infrared fluorescence imaging system (LI-COR).

IP analysis was performed as previously described (Qiu et al., 2014).

**Quantitative real-time PCR**

The primers for qPCR are shown in Supplementary Table S2, and qPCR experiment was performed in an ABI 7300 system. Relative gene expression was calculated using the 2^ΔΔCt method.

**Confocal microscopy**

GMCs were incubated in a glass-bottom culture dish, attacked by sublytic C5b-9 for 40 min, fixed in fresh 4% paraformaldehyde, and blocked with normal goat serum. Subsequently, cells were incubated with C5b-9 Ab at 4°C overnight, and incubated with secondary Cy3-conjugated goat anti-rabbit immunoglobulin G (IgG; Beyotime) and 4', 6-diamidino-2-phenylindole. Confocal images were obtained with an LSM710 confocal microscope (ZEISS).

**Flow cytometry**

GMCs (1 × 10^6) were resuspended in binding buffer containing Annexin V (AV)-FITC and PI or AV-APC and 7-AAD (BD Bioscience). The samples were analyzed on a FACScan flow cytometer (BD Bioscience). The percentage of apoptosis cells in a 10000-cell cohort was determined by flow cytometry.

**In vivo ubiquitination assay**

For analysis of ATF3 ubiquitination, GMCs were transfected with shCTR or shEgr-1. After 48 h, the cells were treated with sublytic C5b-9 with or without 10 μM MG132 for 3 h (Zhang et al., 2014b). Cell lysates were then subjected to IP using anti-ATF3 or anti-ubiquitin Ab.

**mRNA stability detection**

GMCs transfected with shEgr-1 or shCTR were treated with sublytic C5b-9 for 1 h. The cellular transcription was then arrested by 5 μM actinomycin D treatment in the presence of 4% NHS for the indicated time (Park et al., 2013). The ATF3 mRNA was measured by qPCR.

**Genome walking**

Genome walking was carried out by using a TAKARA Genome Walking kit. Briefly, the primary PCR used the outer adaptor primer 2 (AP2) provided in the kit and an inner gene-specific primer1 (SP1) with GMC genome DNA as the template. The primary PCR mixture was diluted (1:1000) and used as a template for the second round nested PCR with AP2 and SP2, and then third round nested PCR with AP2 and SP3. Finally, the third PCR band was purified and sequenced. The detail specific primers are as follows: SP1, 5′-TCACGCTCAGTGCAAAGAGAAGTTCC-3′; SP2, 5′-CGACGGACTTGGTGGCAGAAC-3′; SP3, 5′-CCAGGTCAGAGCCGCCCTAGA-3′.
Luciferase reporter experiments

The full-length or different deletion promoter reporters of ATF3, Gadd45α, Gadd45β, or Gadd45γ were transfected into GMCs with or without different plasmids. A pRL-SV40 vector was always included in each transient transfection. At 48 h after transfection, GMCs were treated or untreated with sublytic C5b-9 for 3 h. The luciferase activity was measured (Liu et al., 2012) using a dual-luciferase reporter assay kit from Promega.

ChIP, ChID, and Re-ChIP

ChIP was performed by using antibodies against Egr-1, ATF3, and preimmune IgG as previously described (Zhang et al., 2014a). For ChID, ATF3-ChIP material from GMCs was released from the agaroses, and the supernatant was purified and amplified by qPCR. For Re-ChIP, ATF3-ChIP material from GMCs was released from the agaroses, and the elution was split into two aliquots for the second round of IP using anti-Ac-K Ab or anti-p300 Ab. Preimmune IgG was a mock control, and the precipitation was purified and amplified by qPCR (Liu et al., 2011). The primers are shown in Supplementary Table S2.

Mass spectrometry

Mass spectral analyses were performed at Center of Hygienic Analysis and Detection of Nanjing Medical University. Anti-ATF3 immunoprecipitates from GMCs were electrophoresed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed with silver staining. Protein bands were excised and collected, subjected to trypsin digestion at 37°C for 16 h. The resulting peptides were purified and analyzed on the mass spectrometer (ULTRAFLX-II, BRUKER).

Renal histological examination

For LM, paraffin-embedded renal sections (4 μm) on day 7 after Thy-1N induction were stained with H&E. The number of glomerular cells was quantified from counts of positive-stained nuclei in a double-blinded manner by two independent observers. A hundred glomerular cross-sections from each rat were examined. For EM, ultrathin sections of renal tissues at 3 h or on day 7 were stained with uranyl acetate and lead citrate, and ultrastructural changes were observed with Tecnai G2 Spirit Bio TWIN (FEI).

TUNEL analysis in vivo

TUNEL staining was performed according to the manufacturer’s instructions (Roche). The TUNEL-positive nuclei number in 100 glomerular cross-sections was counted in a double-blinded manner under an Imager A1 fluorescence microscope (ZEISS).

Urine protein detection

Urine of all rats was collected on day 7. The urinary protein contents (mg per 24 h) were measured by the total protein UC FS (DiaSys Diagnostic Systems). Each sample was assayed in triplicates.

Statistical analysis

Data are presented as mean ± SE of three independent experiments. One-way ANOVA was used to determine significant differences among groups, while comparisons between two groups were analyzed by the t test. A value of P < 0.05 was considered statistically significant.

Supplementary material

Supplementary material is available at Journal of Molecular Cell Biology online.

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