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**ENHANCED EPITHELIAL-TO-MESENCHYMAL TRANSITION
ASSOCIATED WITH LYSOSOME DYSFUNCTION IN PODOCYTES:
ROLE OF P62/SEQUESTOSOME 1 AS A SIGNALING HUB**

by

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LIST OF ABBREVIATIONS

Baf	bafilomycin A1
EMT	epithelial-to-mesenchymal transition
FSP-1	fibroblast-specific protein-1
P-cad	P-cadherin
Sp-1	spaudin-1
ZO-1	zonula occludens-1
CDK1	cyclin-dependent kinase 1
NF κ B	nuclear factor kappa-light-chain-enhancer of activated B cell
Nrf 2	nuclear factor erythroid 2-related factor 2
TGF- β 1	transforming growth factor- β 1
p62	Sequestosome 1
siRNA	small interfering RNA
V-ATPase	vacuolar- type H ⁺ -ATPase
LC3B	light chain 3B
α -SMA	α -smooth muscle actin
RO	RO-3306
Bor	bortezomib
RPM	rapamycin
7-keto	7-ketocholesterol

ABSTRACT

ENHANCED EPITHELIAL-TO-MESENCHYMAL TRANSITION ASSOCIATED WITH LYSOSOME DYSFUNCTION IN PODOCYTES: ROLE OF P62/SEQUESTOSOME 1 AS A SIGNALING HUB

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Virginia Commonwealth University, 2015

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Autophagy is of importance in the regulation of cell differentiation and senescence in podocytes, the highly differentiated glomerular epithelial cells. It is possible that derangement of autophagy under different pathological conditions activates or enhances Epithelial-to-Mesenchymal Transition (EMT) in podocytes, resulting in glomerular sclerosis. To test this hypothesis, the present study produced lysosome dysfunction by inhibition of vacuolar- type H^+ -ATPase (V-ATPase) to test whether deficiency of autophagic flux enhances EMT in podocytes. By Western blot analysis, inhibition of lysosome function by V-ATPase inhibitor or its siRNA was found to induce a significantly enhanced EMT in cultured podocytes, as shown by marked decreases in P-cadherin (P-cad) and zonula occludens-1 (ZO-1) as epithelial markers and simultaneous increases in the mesenchymal markers, fibroblast specific protein-1 (FSP-1) and α -smooth muscle actin (α -SMA). These changes in EMT markers were confirmed by confocal microscopy. This enhancement was accompanied by deficient autophagic flux, as demonstrated by remarkable increases in LC3B-II levels and accumulation of p62/Sequestosome 1 (p62) regardless of whether the autophagosome formation was stimulated or not. However, inhibition of the autophagosome formation using spautin-1 (Sp-1) significantly attenuated both

enhancement of EMT and deficiency of autophagic flux. To explore the mechanisms by which deficient autophagic flux enhances EMT, we tested the role of accumulated p62 as a signal hub in this process. Neither the nuclear factor erythroid 2-related factor 2 (Nrf2) or nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κBs) pathway of p62 regulation contributed to enhanced EMT. However, inhibition of cyclin-dependent kinase 1 (CDK1) activity reduced phosphorylation of p62 and enhanced EMT in podocytes similar to lysosome dysfunction. Given that the lack of phosphorylated p62 leads to a faster exit from cell mitosis, enhanced EMT associated with lysosome dysfunction may be attributed to accumulation of p62 and associated reduction of p62 phosphorylation.

CHAPTER ONE

INTRODUCTION

Podocytes represent a major cellular component of the renal filtration barrier, and damage and loss of these cells are critical for the progression of glomerular nephropathy and renal failure. Recent studies have proposed two major hypotheses to account for the loss of podocytes under different pathological conditions. The first hypothesis emphasizes the importance of podocyte depletion resulting from injury-induced cell death as a causative factor in the onset of proteinuria and glomerular sclerosis [1-3]. According to this hypothesis, the reduced podocyte number in glomeruli is attributed to the apoptotic death of these cells. The second hypothesis proposes that the podocyte loss occurs from an epithelial to mesenchymal transition (EMT) induced by cell injury. Indeed, podocytes have been shown to undergo EMT in response to various injurious stimuli including transforming growth factor- β 1 (TGF- β 1) [4, 5], high glucose [5, 6], homocysteine [7-11], and adriamycin [5].

1.1 Epithelial-to-Mesenchymal Transition

It has been shown that the EMT process is characterized by loss of its epithelial features as indicated by reduced level of podocyte-derived proteins such as nephrin, P-cadherin (P-cad), and zonula occludens-1 (ZO-1) and by acquiring mesenchymal features such as increases in the expression of desmin, fibroblast-specific protein-1 (FSP-1), and α -smooth muscle actin (α -SMA) [10]. This podocyte phenotype change may lead to disruption of its delicate architecture, impairing glomerular filtration membrane function and triggering glomerular injury and sclerosis [12, 13]. However, it remains poorly understood how the process of EMT in podocytes is activated and regulated in response to different pathological stimuli.

1.1.1 The concept of epithelial-to-mesenchymal transition

Metazoans are mainly composed of two cells types, epithelial and mesenchymal, which differ both morphologically and functionally. Epithelial cells are closely attached to each other by intercellular adhesion complexes in their lateral membranes to form coherent layers. In addition, they display apico-basal polarity and characteristic basally localized basement membrane that separates the epithelium from other tissues. In contrast, mesenchymal cells are nonpolarized and lack of intercellular junctions, which enables them move individually throughout the extracellular matrix [40]. EMT is the conversion of adherent epithelial cells into individual migratory cells that can invade the extracellular matrix [41], leading to the disruption of intercellular adhesion complexes and loss of characteristic apico-basal polarity of the epithelial cells [42-44]. Because of cytoskeletal changes, the epithelial cells leave the epithelium and migrate individually, which initially occur by formation of apical constrictions and disorganization of the basal cytoskeleton [45, 46]. Simuultaneously, protease activity leads to collapse of basement membrane and cell ingression [47]. Finally, these cells acquire migratory and invasive properties that capacitate them to migrate through the extracellular matrix.

1.1.2 Pathogenic role of epithelial-to-mesenchymal transition in nephropathy

Emerging evidence has established EMT as a major mechanism of tubulointerstitial fibrosis and glomerulosclerosis [48-50]. EMT typically occurs in response to a number of environmental stresses and associated cytokine/growth factor stimuli such as mechanical stretch [52], cyclosporine treatment [53], exposure to advanced glycation end products (AGE consequence of hyperglycaemia) [54], hypoxia [55], hyperhomocysteinemia, oxidative stress [56], aldosterone [57], activated monocyte supernatant, interleukin-1 [58], and oncostatin M treatment as well as the culturing of cells on collagen I [59]. Our previous study reveals that the normal expression of

CD38 importantly contributes to the differentiation and function of podocytes and defect of this gene expression may be a critical mechanism inducing EMT and consequently resulting in glomerular injury and sclerosis [51]. However, the normal regulation of EMT in the kidney, in particular in glomerular podocytes, is still poorly understood.

1.2 Autophagy

In this regard, previous studies have demonstrated that podocyte differentiation and maturation are highly dependent upon normal autophagy [12, 14]. Given that EMT involves a fundamental change in differentiation state, it is plausible that deficient autophagy contributes to the activation or enhancement of EMT. It is well known that autophagy is a cell survival mechanism responsible for the degradation of long-lived or damaged proteins and excessive or dysfunctional cell organelles [13, 15, 16]. Under physiological conditions, autophagy functions in a continuous, reparative way to maintain normal cellular homeostasis. In addition to the formation of autophagosomes, autophagy also includes the autophagic flux consisting of the fusion of autophagosomes to lysosomes and the lysosomal enzymatic degradation of these autophagic substances. This autophagic flux is determined by lysosome function and therefore the normal lysosome function plays a critical role in maintenance of autophagic process, keeping podocytes in a differentiated and functional status. Indeed, we have recently reported that the regulation of lysosome function importantly contributes to autophagic flux or autophagy maturation in mouse podocytes and that lysosome dysfunction or injury due to derangement of its regulatory mechanisms resulted in deficiency of autophagic flux and consequent EMT [12, 17]. It is now imperative to address how a deficient autophagic flux associated with lysosome dysfunction activates or enhances podocyte EMT.

1.2.1 Selective autophagy

It is unclear how much basal autophagy contributes to macromolecule synthesis and energy production in the steady state by supplying amino acids, glucose, and free fatty acids. Nevertheless, basal autophagy acts as the quality-control machinery for cytoplasmic components, and it is crucial for homeostasis of various postmitotic cells, such as neurons and hepatocytes. Although this quality control could be partially achieved by nonselective autophagy, increasing evidence indicates that “selective” autophagy degrades specific proteins, organelles, and invading bacteria. Selective autophagy occurs constitutively and can also be induced in response to cellular stresses [60].

One of the best characterized substrates of selective autophagy is p62, which is also known as sequestosome 1/SQSTM1. p62 is an ubiquitously expressed cellular protein, which is conserved in animals but not in plants and fungi. p62 directly interacts with LC3 (microtubule-associated protein light chain 3) on the isolation membrane through the LC3-interacting region. Subsequently, p62 is incorporated into the autophagosome and then degraded [61, 62]. Due to the presence of the C-terminal ubiquitin associated (UBA) domain of p62, in addition to the binding capacity to LC3, p62 is thought to be a receptor for ubiquitinated cargos including ubiquitinated aggregates, damaged mitochondria, ubiquitinated midbody rings, ubiquitin-tagged peroxisomes, ubiquitinated microbes, ribosomal proteins, and virus capsid protein, to deliver them to the autophagosomes [64–66]. p62, as an adaptor protein, mediates the degradation of ubiquitinated cargos through their interaction with ubiquitin. This selective autophagy could be regulated by posttranslational modification of the adaptors. Impairment of autophagy is accompanied by accumulation of p62. This leads to the formation of large aggregates, which include p62 and ubiquitin [63].

1.2.2 The role of p62 as a signaling hub

In addition to the definitive role of autophagy in starvation adaptation, analyses of a large number of mouse lines with tissue-specific deletions of Atg have demonstrated that loss of autophagy causes various life-threatening diseases in spite of the nutritional status [60]. However, the pathoetiology cannot be simply accounted for by impairment of basal and constitutive autophagy as a house cleaner: intracellular global turnover. Suppression of autophagy is always accompanied by massive accumulation of a selective substrate for autophagy; p62. In addition to its crucial role as an assembly factor for ubiquitinated proteins and organelles, p62 functions as a signaling hub in various transduction pathways, such as NF- κ B signaling, activation of apoptosis, and response to environmental stress [32]. Whereas these signals are enhanced by aggregation of signaling complex through p62, selective turnover of p62 via autophagy might shut them off. If this is true, impaired autophagy and/or abnormal expression of p62 should be accompanied by persistent activation of these signaling pathways, leading to serious diseases, such as tumorigenesis.

1.2.3 Vacuolar- type H⁺-ATPase and autophagosome-lysosome fusion

V-ATPases are ubiquitous ATPdependent proton pumps, organized in multisubunit complexes, which couple the energy released from ATP hydrolysis with the active extrusion of protons from the cytoplasm into the lumen of organelles or to the extracellular space [67]. The enzyme is composed of a peripheral domain (V₁) that carries out ATP hydrolysis and an integral domain (V₀) responsible for proton transport [68]. V-ATPase is localized in organelles of the central vacuolar system such as coated vesicles, endosomes, lysosomes, chromaffin granules, and Golgi apparatus, and plays an important role by maintaining the acidic environment in these compartments [71].

In the process of autophagy, membrane structures called isolation membranes or phagophores appear, segregate parts of the cutoplasm, and form autophagosomes. The newly formed autophagosomes (early autophagosomes) fuse with endosomes or prelysosomes, and become a more advanced stage of autophagosomes (late autophagosomes or amphisomes) of acidic lyminal pH [72-74]. The autophagosomes then acquire hydrolytic enzymes by fusion with lysosomes, and are transformed into mature autolysosomes in which degradation of the content proceeds [73, 75, 76]. In previous studies, it has been proved that the internal acidification of vesicles is essential to vesicular transport [77-81]. Furthermore, the activity of V-ATPase is required for the maturation of endosomes and fusion between endosomes and lysosomes [77, 79]. Therefore, the inhibition of V-ATPase is considered to block autophagic flux by disturbing lysosome movement and fusion between autophagosome and lysosome.

Bafilomycin A1 (Baf) is known as a specific and potent inhibitor of V-ATPase by preventing the rotation of subunit a [69] and subunit c [70], which are subunits of V_0 . It has been reported that the obvious block of autophagosome-lysosome fusion is an indirect result of the acidification defect, which follows the immediate result of Baf treatment [71, 82]. For this reason, we will use Baf as the inhibitor of V-ATPase to produce the condition of lysosome dysfunction in our experiments.

1.3 Aims of the study

The hypothesis to be tested in the present study states that: lysosome dysfunction may induce podocyte EMT due to the accumulation of autophagosome, p62 aggregation, and activation of associated signaling pathways such as Nrf2-mediated redox sensing, NF- κ B-

dependent transcriptional regulation, and cyclin-dependent kinase 1 (CDK1)–mediated phosphorylation of p62.

The specific aims are:

1. To determine whether inhibition of lysosome function by V-ATPase inhibitor and its siRNA induces EMT in podocytes by observations of changes in epithelial and mesenchymal markers.
2. To determine whether inhibition of lysosome function leads to accumulation of autophagosome and aggregation of p62 in podocytes, a typical deficiency of autophagic flux, which may contribute to activation or enhancement of podocyte EMT.
3. To determine the mechanisms by which lysosome dysfunction leads to EMT by testing the role of p62-related signaling pathways. Our results demonstrate that p62 accumulation and associated reduction in phosphorylation of p62 may be a novel mechanism switching on EMT associated with lysosome dysfunction and autophagic flux deficiency in podocytes.

CHAPTER TWO

GENERAL METHODS

2.1 Cell culture

Conditionally immortalized mouse podocytes, kindly provided by Dr. Klotman PE (Division of Nephrology, Department of Medicine, Mount Sinai School of Medicine, New York, NY, USA) were cultured on collagen I-coated flasks or plates in RPMI 1640 medium supplemented with recombinant mouse interferon- γ at 33°C. After differentiation at 37°C for 10-14 days without interferon- γ , podocytes were used for proposed experiments. The concentrations used for all protocols were decided based on preliminary dose response data showing that 5 nM bafilomycin A1 (Baf) had stable effects on activation of EMT in podocytes.

2.2 Immunofluorescence microscopy

Double-immunofluorescence staining was performed using cultured podocytes on cover slips. After fixation, the cells were incubated with rabbit anti-podocin 1: 200 (Sigma, St. Louis, MO, USA), which was followed by incubation with Alexa-488-labeled donkey anti-rabbit secondary antibody. Then, goat anti-FSP-1 (1:50 dilution), goat anti-ZO-1 (1:50 dilution) (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), goat anti-P-cadherin (1:25 dilution), or mouse anti- α -SMA (1:300 dilution) (R&D system, Minneapolis, MN, USA) were added to the cell slides and then incubated overnight at 4°C. After washing, the slides were incubated with corresponding Alexa-555-labeled secondary antibodies and then mounted and analyzed by confocal laser scanning microscopy (Fluoview FV1000, Olympus, Japan). These double staining

experiments were performed to observe the relationship between podocin production and EMT changes during Baf incubation.

In addition to double staining and confocal microscopy, several groups of podocytes were used for quantitative analysis of expression of markers of EMT, autophagosomes and lysosomes by fluorescence microscopy. In these experiments, podocytes on cover slips were fixed in 4% paraformaldehyde for 20 minutes. After rinsing with phosphate-buffered saline (PBS), the cells were incubated with rabbit anti-FSP-1 (1:100, Abcam, Cambridge, MA, USA), rabbit anti-ZO-1 (1:50, Invitrogen, Camarillo, CA, USA), rabbit anti-P-cadherin (1:25), mouse anti- α -SMA (1:300, R&D system, Minneapolis, MN, USA) antibodies, rabbit anti-LC3B (Cell Signaling Technology, Beverly, MA, USA), or rat anti-LAMP1 (Novus Biologicals, St. Louis, MO, USA). After washing, the slides were incubated with the corresponding Alexa 488-labeled secondary antibodies for 1 h at room temperature. After mounting with DAPI-containing mounting solution, the slides were observed under a fluorescence microscope and photos were taken and analyzed.

2.3 Western blot analysis

Western blot analysis was performed as we described previously [10]. In brief, homogenates from cultured podocytes were prepared using sucrose buffer containing protease inhibitors. After boiling for 5 min at 95°C in a 5× loading buffer, total cell protein (20 μ g) was subjected to SDS-PAGE, transferred onto a PVDF membrane and blocked by solution with dry milk. Then the membrane was probed with primary antibodies against anti-ZO-1 (1:1000, Invitrogen), anti-P-cadherin (1: 1000, R&D System), anti- α -SMA (1:5000, R&D System), anti-

FSP-1 (1:1000, Abcam), anti-LC3B (1:1000, Cell Signaling Technology), anti-LAMP1 (1:1000, Novus Biologicals), mouse anti-p62 (1:5000, Abcam, Cambridge, MA, USA), rabbit anti-phospho-p62 (1:1000, Cell Signaling Technology, Beverly, MA, USA) or anti- β -actin (1:5000, Santa Cruz Biotechnology) overnight at 4 °C followed by incubation with horseradish peroxidase-labeled IgG (1:5000). The immunoreactive bands were detected by chemiluminescence methods and visualized on Kodak Omat X-ray films. Densitometric analysis of the images obtained from X-ray films was performed using the Image J software (NIH, Bethesda, MD, USA).

2.4 siRNA transfection

Vacuolar H⁺-ATPase (V-ATPase) siRNA (Santa Cruz Biotechnology, Dallas, TX, USA), Nrf2 siRNA (Santa Cruz Biotechnology, Dallas, TX, USA), NF κ B siRNA (Cell Signaling Technology, Beverly, MA, USA), and CDK1 siRNA (Santa Cruz Biotechnology, Dallas, TX, USA) were confirmed to be effective in silencing the target genes in different cells by the companies. The scrambled RNA (Qiagen, Valencia, CA, USA) was confirmed as non-silencing double-strand RNA and used as the control in the present study. Podocytes were serum starved for 12 h and then transfected with siRNA or scrambled RNA using siLentFect Lipid Reagent (Bio-Rad, Hercules, CA, USA). After 24 h of incubation at 37°C, the medium was changed, and Baf (5 nM) was added into the medium for indicated time span in different protocols.

2.5 Statistical analysis

All of the values are expressed as mean \pm SEM. Significant differences among multiple groups were examined using ANOVA followed by a Student-Newman-Keuls test. χ^2 test was

used to assess the significance of ratio and percentage data. $P < 0.05$ was considered statistically significant.

CHAPTER THREE

Epithelial-to-mesenchymal transition enhanced in mouse podocytes during inhibition of lysosome function

3.1 Enhanced EMT by inhibition of lysosome function in podocytes

To confirm that inhibition of lysosome function enhances EMT in podocytes, Western blot analyses were performed on homogenates from cells before and after treatment with Baf or the siRNA for V-ATPase. As shown in Fig. 1A and 1B, when podocytes were treated with Baf, the epithelial markers P-cad and ZO-1 decreased significantly, while the mesenchymal markers FSP-1 and α -SMA increased markedly. Similarly, V-ATPase siRNA transfection (siv-A) decreased P-cad and ZO-1 but increased FSP-1 and α -SMA significantly in podocytes (Fig. 1C and 1D). It is clear that the ratio of epithelial to mesenchymal markers was significantly reduced during inhibition of lysosome function, suggesting a large enhancement of EMT in podocytes.

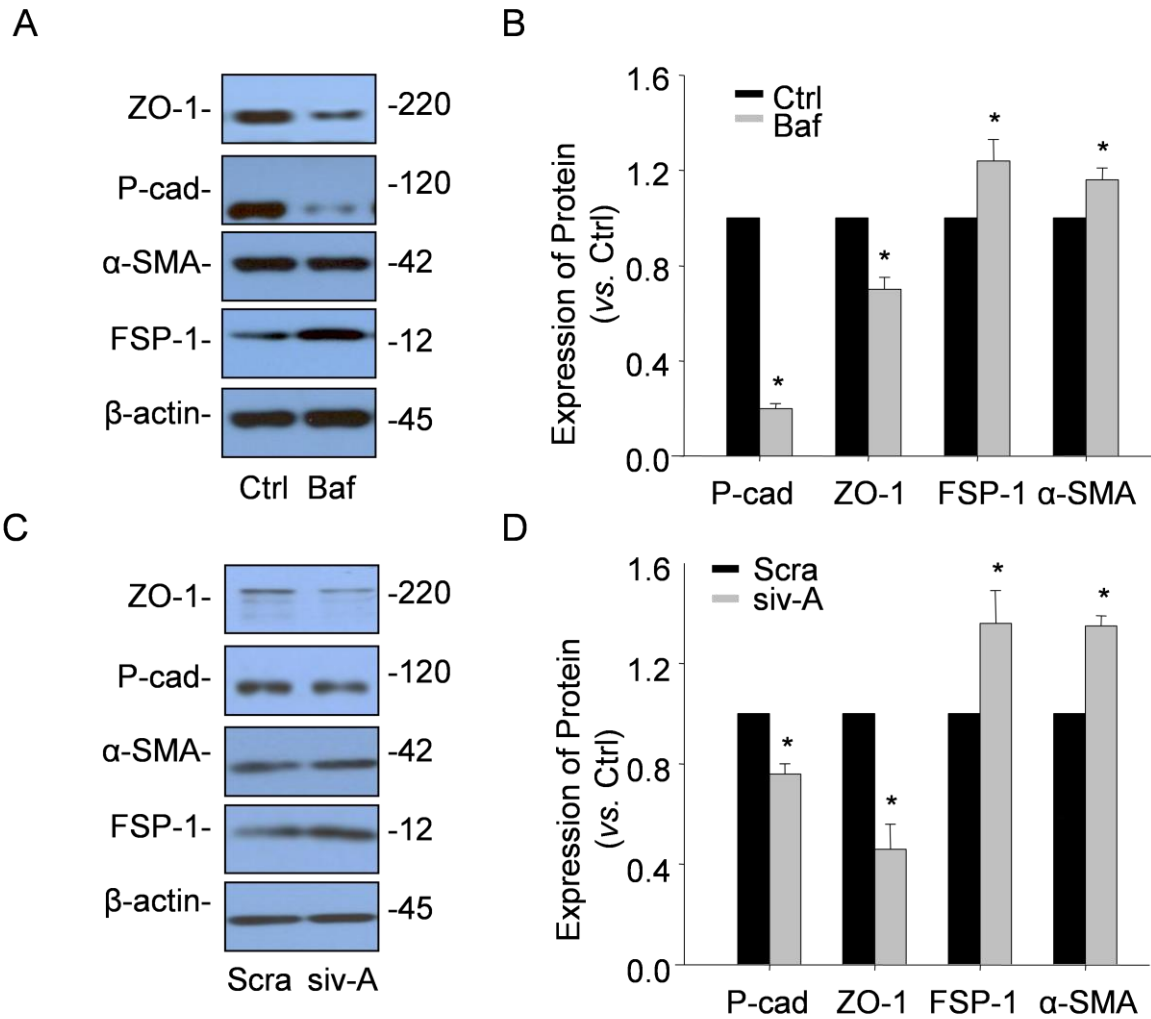


Figure 1. Effects of inhibition of lysosome function on EMT in podocytes. Podocytes were stimulated by Baf (5 nM) or transfected with V-ATPase siRNA (10 nM) for 24 hours. A. Representative gel images showing ZO-1 and P-cadherin as epithelial markers and α -SMA and FSP-1 as mesenchymal markers in different groups. B. Summarized data showing the relative levels of ZO-1 and P-cadherin as epithelial markers and α -SMA and FSP-1 as mesenchymal markers, quantitated as a ratio of detected specific protein band vs. β -actin as loading control (n=3-5). C. Representative gel images showing the expression of ZO-1 and P-cadherin as epithelial markers and the expression of α -SMA and FSP-1 as mesenchymal markers in different groups. D. Summarized data showing the expression of ZO-1 and P-cadherin as epithelial markers and the expression of α -SMA and FSP-1 as mesenchymal markers, quantitated as a ratio of detected specific protein band vs. β -actin as loading control (n=3-5). * P<0.05 vs. Ctrl.

3.2 Confocal microscopy of EMT during inhibition of lysosome function

Using confocal microscopy, the effects of inhibition of lysosome function on EMT in podocytes were further detected. As shown in Fig. 2A, under basal conditions, podocytes were enriched with P-cadherin and ZO-1. After treatment with Baf, both P-cadherin and ZO-1 fluorescent staining were significantly reduced. As shown in the overlaid cell image (OL in left panels of Fig. 2A), there was less co-localization of podocin with ZO-1 or P-cad in Baf-treated podocytes compared with control podocytes. In contrast, FSP-1 and α -SMA increased after the Baf treatment as shown by enhanced co-localization of podocin with both markers (right panels of Fig. 2A). In podocytes transfected with V-ATPase siRNA (siv-A), detected epithelial marker and mesenchymal marker stainings were similar to that shown in Baf-treated podocytes. Decreases in epithelial marker and increases in mesenchymal markers were observed (Fig. 2B).

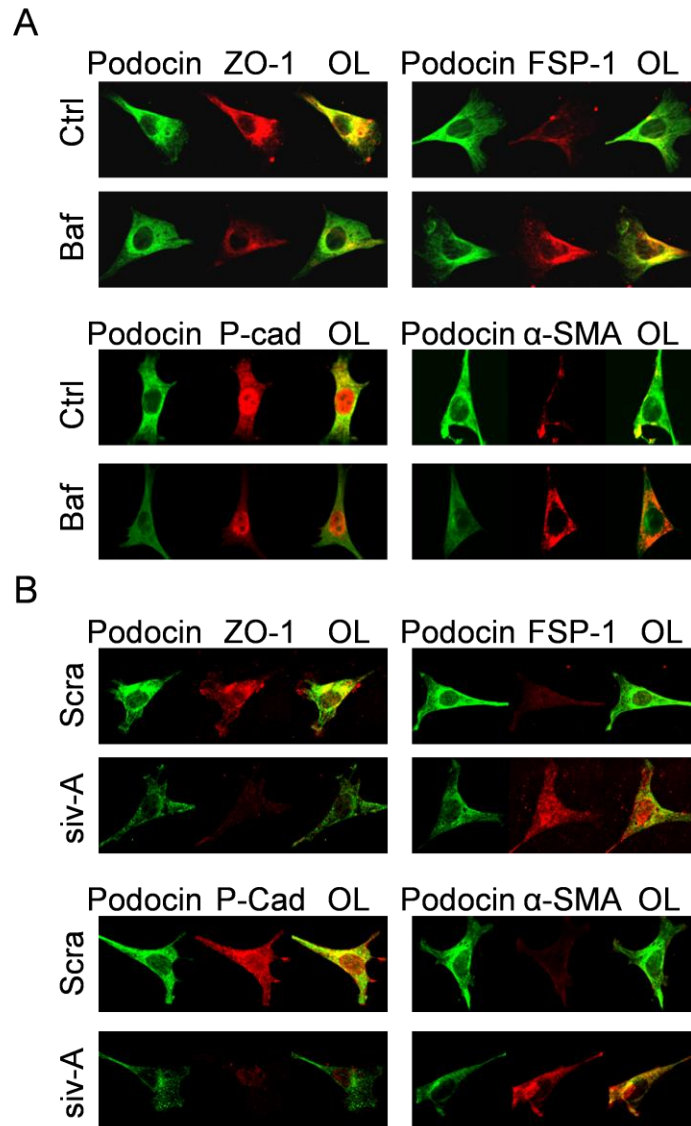


Figure 2. 3.2 Confocal microscopy of EMT during inhibition of lysosome function. A. Images showing double-immunostained podocytes for epithelial markers, P-cadherin and ZO-1 (Alexa 555, red color) or mesenchymal markers, α -SMA and FSP-1 (Alexa 555, red color) with podocyte marker, podocin (Alexa 488, green color) in different groups (n=5). B. Images showing double-immunostained podocytes for epithelial markers, P-cadherin and ZO-1 (Alexa 555, red color) or mesenchymal markers, α -SMA and FSP-1 (Alexa 555, red color) with podocyte marker, podocin (Alexa 488, green color) in different groups (n=4). Ctrl: Control, Baf: Baf, siv-A: V-ATPase siRNA.

CHAPTER FOUR

Contribution of accumulated autophagosome to the enhancement of epithelial-to-mesenchymal transition during inhibition of lysosome function

4.1 Deficiency of autophagic flux during inhibition of lysosome function

Given the role of autophagy in podocyte differentiation, we tested whether the enhancement of EMT observed in response to lysosomal inhibition is associated with deficient autophagy. Fig. 3A shows representative Western blot images using antibodies against LC3B, Lamp-1 and p62. Under basal conditions or after co-treatment with the autophagosome formation inducers, rapamycin or 7-ketocholesterol, Baf treatment resulted in marked increases in the levels of the autophagosome markers, LC3B-II and p62, (autophagosome markers) while having no effect on the lysosomal membrane marker, Lamp-1. . As shown in Fig. 3B , lysosomal inhibition by Baf dramatically increased the ratio of LC3B-II vs. LC3B-I, an effect that was independent of whether autophagosome formation was stimulated. The increased LC3B-II level was accompanied by accumulation of p62 in podocytes (Fig. 3C). However, the Lamp-1 level was not significantly changed by Baf (Fig. 3D). Expression of V-ATPase siRNA produced similar effects on autophagic flux to those of Baf, showing that both LC3B-II and p62 significantly increased in podocytes (data not shown).

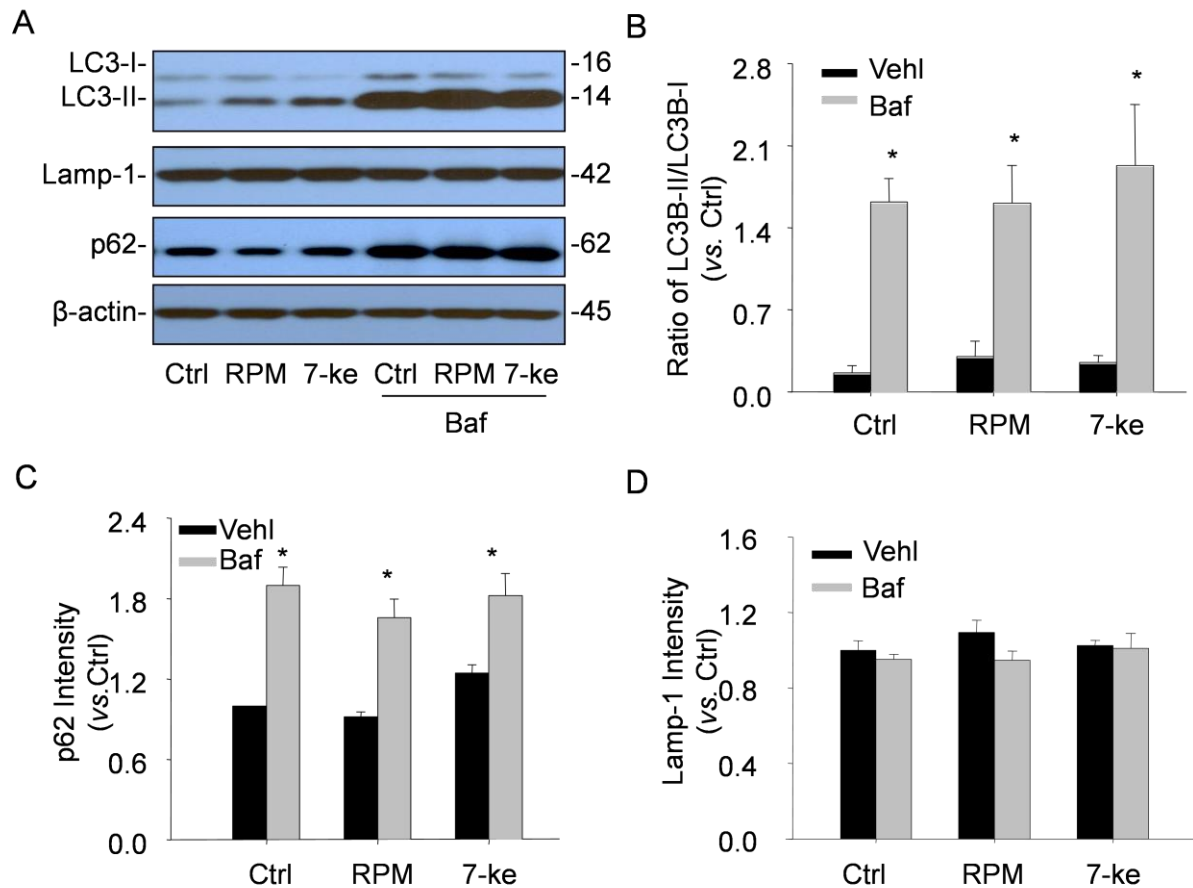


Figure 3. Effects of lysosome function inhibitor on autophagy. Podocytes were stimulated by RPM (20 μ M) or 7-keto (10 μ M) for 24 hours. A. Representative Western blot images showing the levels of LC3B-I, LC3B-II, and p62 as autophagosome markers and Lamp-1 as a lysosome marker in different treatment groups. B. Summarized data showing expression of LC3B-I and LC3B-II, quantitated as a ratio of LC3B-II band over LC3B-I band (n=4). C. Summarized data showing expression of Lamp-1, quantitated as a ratio of detected specific protein band vs. β -actin as loading control (n=4). D. Summarized data showing expression of p62, quantitated as a ratio of detected specific protein band vs. β -actin as loading control (n=4). * P<0.05.

4.2 Attenuation of EMT by Sp-1 inhibition of autophagosome formation

To further determine the role of autophagosome accumulation in podocyte EMT enhanced by inhibition of lysosome function, we examined the effects of the selective autophagosome formation inhibitor, Sp-1, on Baf-enhanced EMT. As shown in Fig. 4A, Western blot analysis showed that Baf dramatically increased the LC3B-II level. In the presence of Sp-1, the effect of Baf on the LC3B-II level was attenuated. As summarized in Fig. 4B, the ratio of LC3B-II vs. LC3B-I was significantly increased by Baf. However, this indicator of Baf-induced autophagosome accumulation was significantly attenuated by Sp-1. Moreover, Baf-induced p62 accumulation was inhibited by Sp-1 as indicated by Western blotting (Fig. 4C). Densitometric analysis showed that inhibition of lysosome function by Baf led to a significant accumulation of p62 in podocytes, which was remarkably lessened by Sp-1 (Fig. 4D). As shown in Fig. 4E, interestingly, enhanced EMT by Baf as shown by decrease in P-cad and increase in α -SMA was obviously attenuated by Sp-1. The ratio of P-cad to α -SMA, an EMT index, was significantly decreased in bafilomycin-treated podocytes. In the presence of Sp-1, P-cadherin to α -SMA ratio was significantly reduced (Fig. 4F).

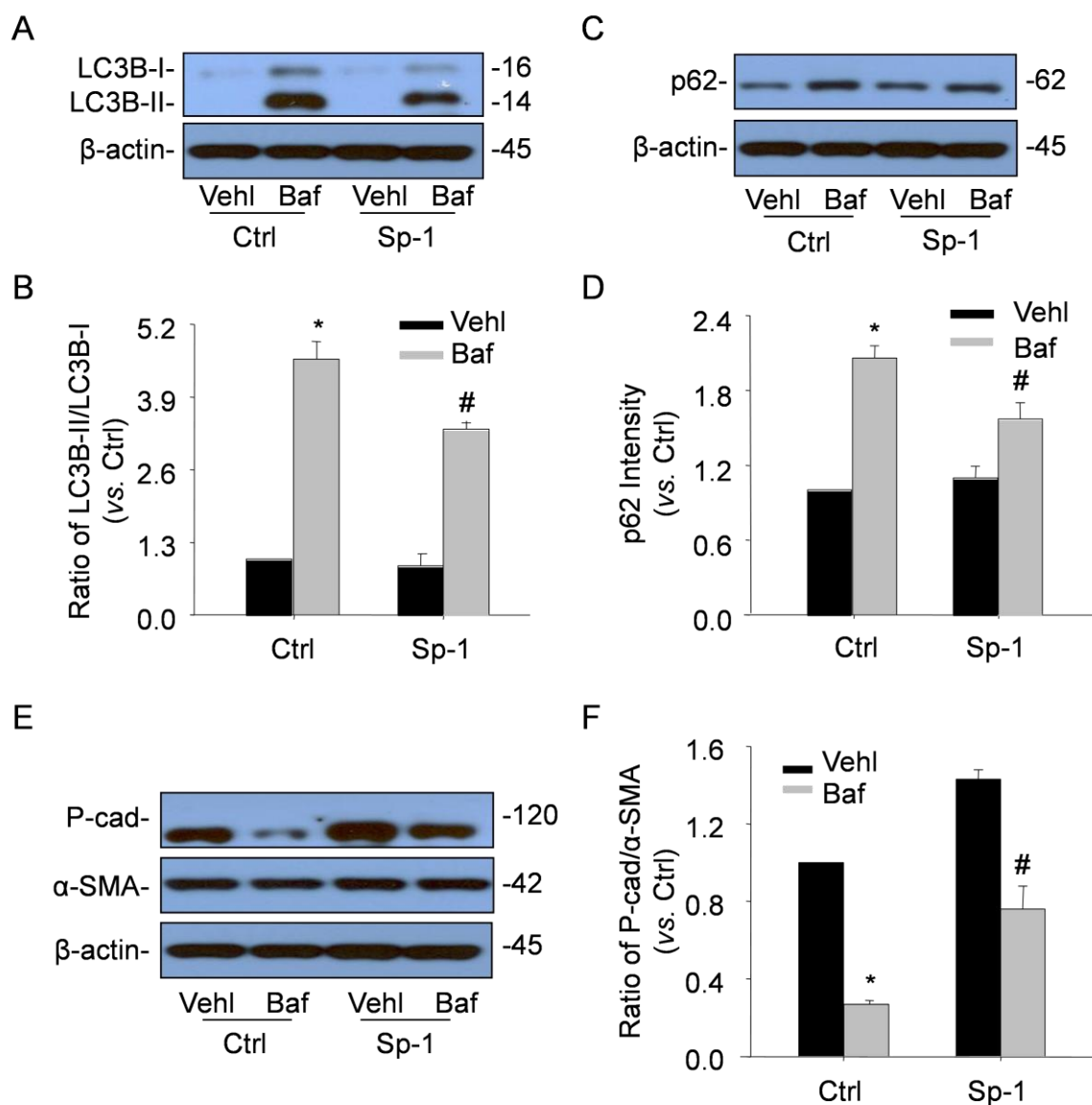


Figure 4. Sp-1 inhibition of autophagosome formation during inhibition of lysosome function. Podocytes were stimulated by Sp-1 (10 μ M) for 24 hours. A. Representative Western blot images showing the expression of LC3B-I and LC3B-II as autophagosome markers in different groups. B. Summarized data showing expression of LC3B-I and LC3B-II, quantitated as a ratio of LC3B-II band over LC3B-I band (n=3). C. Representative gel documents showing the expression of p62 as autophagosome marker in different groups. D. Summarized data showing expression of p62, quantitated as a ratio of detected specific protein band vs. β -actin as loading control (n=3). E. Representative gel documents showing the expression of P-cadherin, α -SMA in different groups. F. Summarized data showing expression of P-cadherin and α -SMA, quantitated as a ratio of P-cadherin band over α -SMA band (n=3-4). * $P < 0.05$ vs. Ctrl; # $P < 0.05$ vs. Baf.

CHAPTER FIVE

No changes in enhanced epithelial-to-mesenchymal transition by Nrf2 gene silencing or inhibition of NF- κ B-mediated transcriptional regulation

5.1 Failure of Nrf2 gene silencing to alter EMT enhanced by inhibition of lysosome function

The role of p62-regulated Nrf2 signaling in the enhancement of EMT by inhibition of lysosome function was examined using siRNA-mediated Nrf2 gene silencing. Fig. 5A shows representative Western blot gel documents illustrating the changes in P-cad, α -SMA and p62 induced by Baf before and after Nrf2 gene silencing (siNrf). The level of P-cad was markedly decreased, while p62 increased in Baf-treated podocytes. These Baf-induced changes in EMT markers in associated with increase in p62 were not altered by Nrf2 gene silencing. As shown in Fig. 5B, Baf significantly decreased the ratio of P-cad and α -SMA, an EMT index. However, Nrf2 gene silencing did not alter the ratio of P-cad to α -SMA. In addition, Baf-induced significant accumulation of p62 was also not altered by Nrf2 siRNA (Fig. 5C).

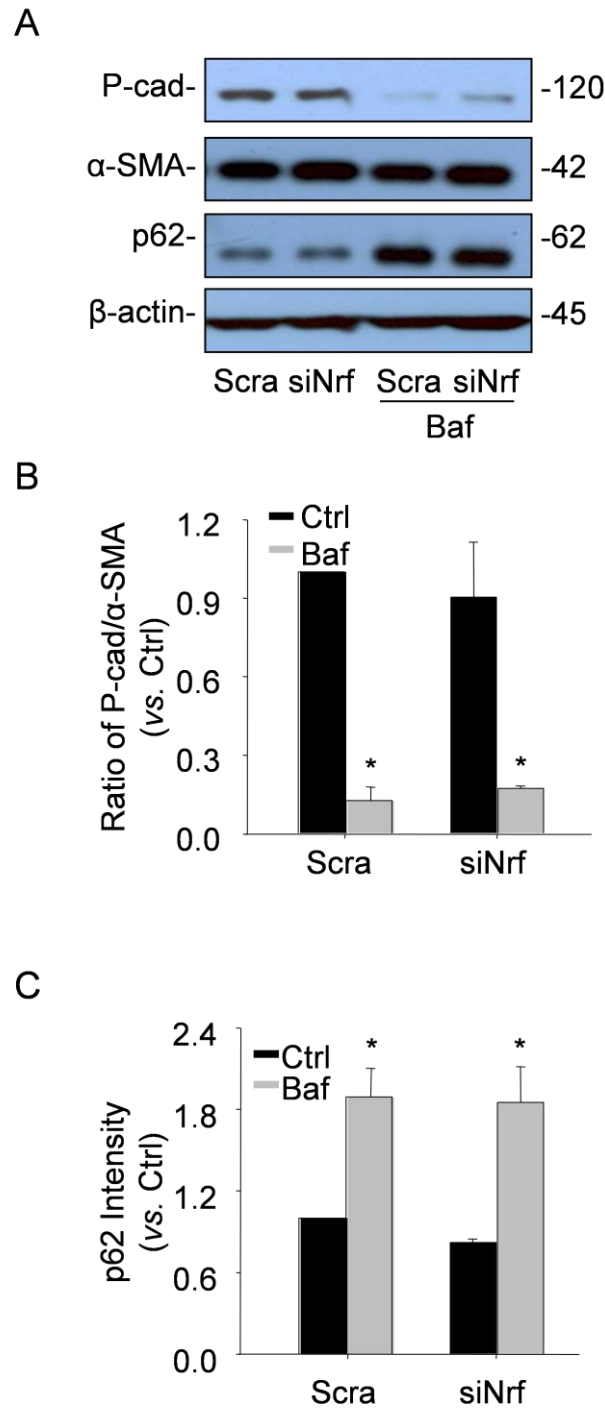


Figure 5. Failure of Nrf2 inhibition by gene silencing to alter EMT enhanced by inhibition of lysosome function. Podocytes were transfected with Nrf2 siRNA (10 nM) for 24 hours. A. Representative Western blot images showing the relative levels of P-cadherin, α -SMA and p62 in different groups. B. Summarized data showing expression of P-cadherin and α -SMA, quantitated as a ratio of P-cadherin band over α -SMA band (n=3-4). C. Summarized data showing expression of p62, quantitated as a ratio of detected specific protein band vs. β -actin as loading control (n=3-4). * P<0.05 vs. Ctrl.

5.2 No changes in enhanced EMT by inhibition of NF- κ B-mediated transcriptional regulation

Given the role of NF- κ B-mediated transcriptional regulation in cell dedifferentiation and its association with p62, we tested whether inhibition of its activity alters podocyte EMT enhanced by inhibition of lysosome function. Representative Western blot gel documents in Fig. 6A showed that the level of P-cad remarkably decreased, but p62 was increased in Baf-treated podocytes. These Baf-induced changes in EMT markers as well as p62 were same before and after pharmacological inhibition of NF- κ B activity. As summarized in Fig. 6B, Baf significantly decreased the ratio of P-cad vs. α -SMA, the EMT index, which was not altered by inhibition of NF- κ B activity. Furthermore, Baf-induced significant accumulation of p62 remained same even though NF- κ B activity was inhibited (Fig. 6C). We also inhibited NF- κ B-mediated transcriptional regulation by gene silencing to further confirm whether this transcription factor is involved in Baf-enhanced EMT. As shown in Fig. 6D, 6E and 6F, the effect of NF- κ B gene silencing (siNF- κ B) on Baf-enhanced EMT was similar to its pharmacological inhibition, without effects on decreases in the ratio of P-cad vs. α -SMA and p62 accumulation in podocytes.

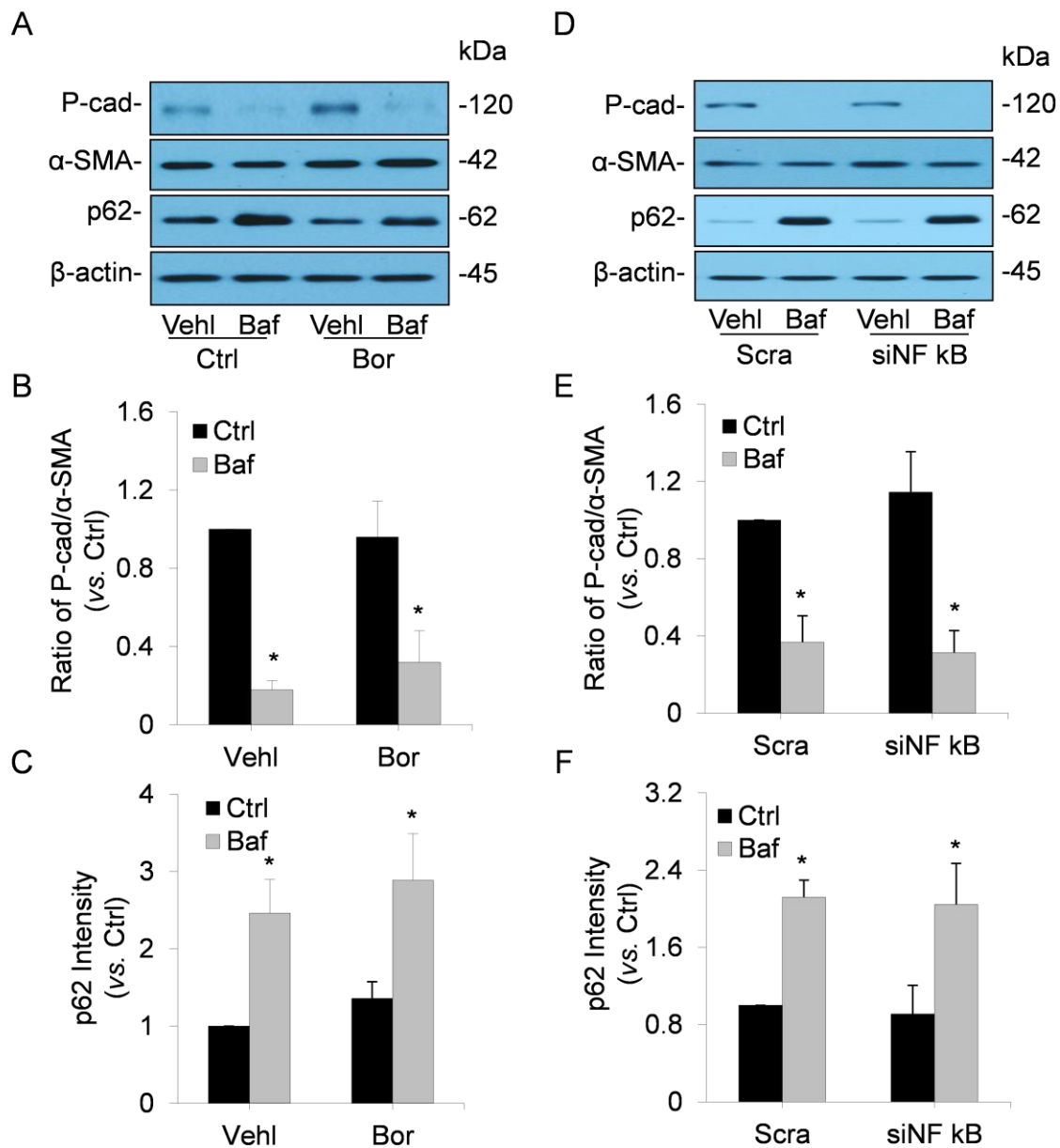


Figure 6. No changes in enhanced EMT by inhibition of NF- κ B-mediated transcriptional regulation. Podocytes were stimulated by Bor (1 nM) or transfected with NF κ B siRNA (10 nM) for 24 hours. A. Representative Western blot images showing relative levels of P-cadherin, α -SMA and p62 in different groups. B. Summarized data showing expression of P-cadherin and α -SMA, quantitated as a ratio of P-cadherin band over α -SMA band (n=5). C. Summarized data showing expression of p62, quantitated as a ratio of detected specific protein band vs. β -actin as loading control (n=5). D. Representative gel documents showing the expression of P-cadherin, α -SMA and p62 in different groups. E. Summarized data showing expression of P-cadherin and α -SMA, quantitated as a ratio of P-cadherin band over α -SMA band (n=5). F. Summarized data showing expression of p62, quantitated as a ratio of detected specific protein band vs. β -actin as loading control (n=4). * P<0.05 vs. Ctrl.

CHAPTER SIX

Contribution of p62 phosphorylation reduction to lysosome function inhibition-induced epithelial-to-mesenchymal transition

6.1 Effects of CDK1 inhibition on EMT enhancement induced by lysosome dysfunction

We also tested whether inhibition of CDK1 expression and activity alters Baf-induced enhancement of EMT in podocytes. It has been reported that phosphorylation of p62 by CDK1 regulates exit from cell cycle or cell arrest during cell mitosis and that reduced phosphorylation of p62 leads to a faster exit from cell mitosis, controlling cell dedifferentiation and tumorigenesis [19]. In Fig. 7, panel A presents representative immunoblots showing the effect of the CDK1 inhibitor, RO-3306 (RO), a quinolinyl thiazolinone derivative, on the Baf-induced effects in podocytes. Similar to Baf, RO treatment alone also decreased the level of P-cad and increased α -SMA, resulting in a significant decrease in the P-cad to α -SMA ratio (Fig. 7B). However, unlike Baf, treatment with RO did not significantly affect p62 (Fig. 7A and 7C). In co-treated cells, RO did not significantly alter the effect of Baf on either the EMT markers or p62 (Fig. 7A-C). To further confirm the role of CDK1 inhibition in enhancement of EMT, we used its siRNA to test whether gene silencing of CDK1 alters EMT. As shown in Fig. 7D, 7E and 7F, CDK1 siRNA (siCDK1) had a nearly identical profile of effects to those of the chemical inhibitor. The siRNA treatment alone decreased the ratio of P-cad to α -SMA but had no effect on the Baf-induced changes in EMT markers or p62.

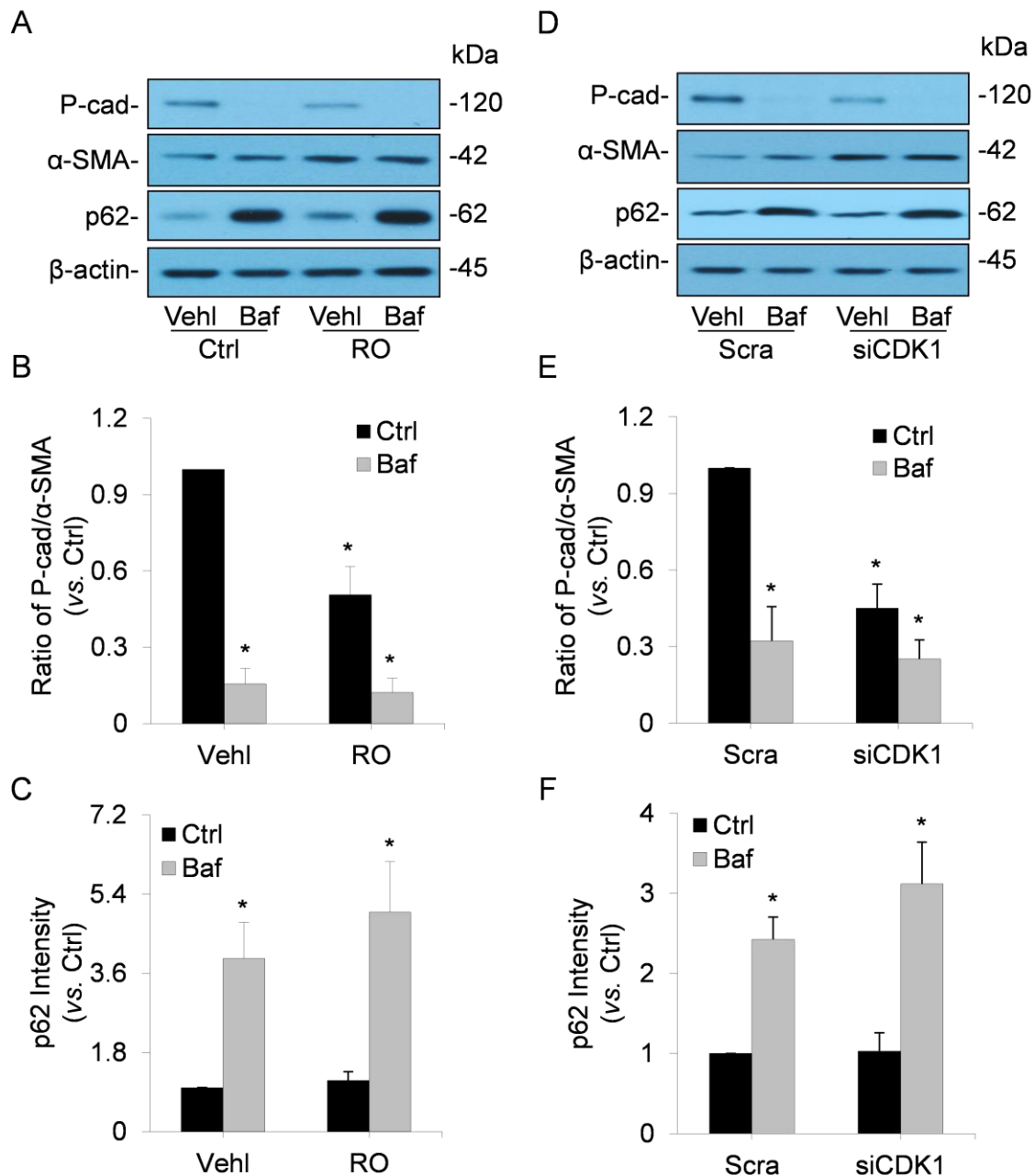


Figure 7. Effects of CDK1 inhibition on EMT enhancement induced by lysosome dysfunction. Podocytes were stimulated by RO (10 μ M) or transfected with CDK1 siRNA (10 nM) for 24 hours. A. Representative Western blot images showing the expression of P-cadherin, α -SMA and p62 in different groups. B. Summarized data showing expression of P-cadherin and α -SMA, quantitated as a ratio of P-cadherin band over α -SMA band (n=5). C. Summarized data showing expression of p62, quantitated as a ratio of detected specific protein band vs. β -actin as loading control (n=9). * P<0.05 vs. Ctrl. D. Representative gel documents showing the expression of P-cadherin, α -SMA and p62 in different groups. E. Summarized data showing expression of P-cadherin and α -SMA, quantitated as a ratio of P-cadherin band over α -SMA band (n=5). F. Summarized data showing expression of p62, quantitated as a ratio of detected specific protein band vs. β -actin as loading control (n=7). * P<0.05 vs. Ctrl.

6.2 *Reduction of p62 phosphorylation during inhibition of lysosome function*

Further experiments were designed to determine whether reduced p62 phosphorylation occurs due to inhibition of lysosome function. Western blot analyses showed that the CDK1 inhibitor, RO, markedly decreased phosphorylated p62 even though it had no effect on the total p62 level in podocytes (Fig. 8A). In contrast to the strong effect of Baf on p62 accumulation, it had little effect on phosphorylation of p62. The calculated ratio of phosphorylated p62 to total p62 was reduced significantly by both RO and Baf, suggesting that the relative reduction of phosphorylated p62 may be involved in the control of EMT (Fig. 8B). We also conducted additional experiments to silence CDK1 gene in order to confirm the role of decreased phosphorylated p62 in podocyte EMT. As presented in Fig. 8C, similar to RO, CDK1 siRNA reduced phosphorylated p62, but had no effects on total p62 level. As summarized in Fig. 8D, the treatment with CDK1 siRNA reduced the ratio of phosphorylated p62 to total p62, similar to the effects of Baf.

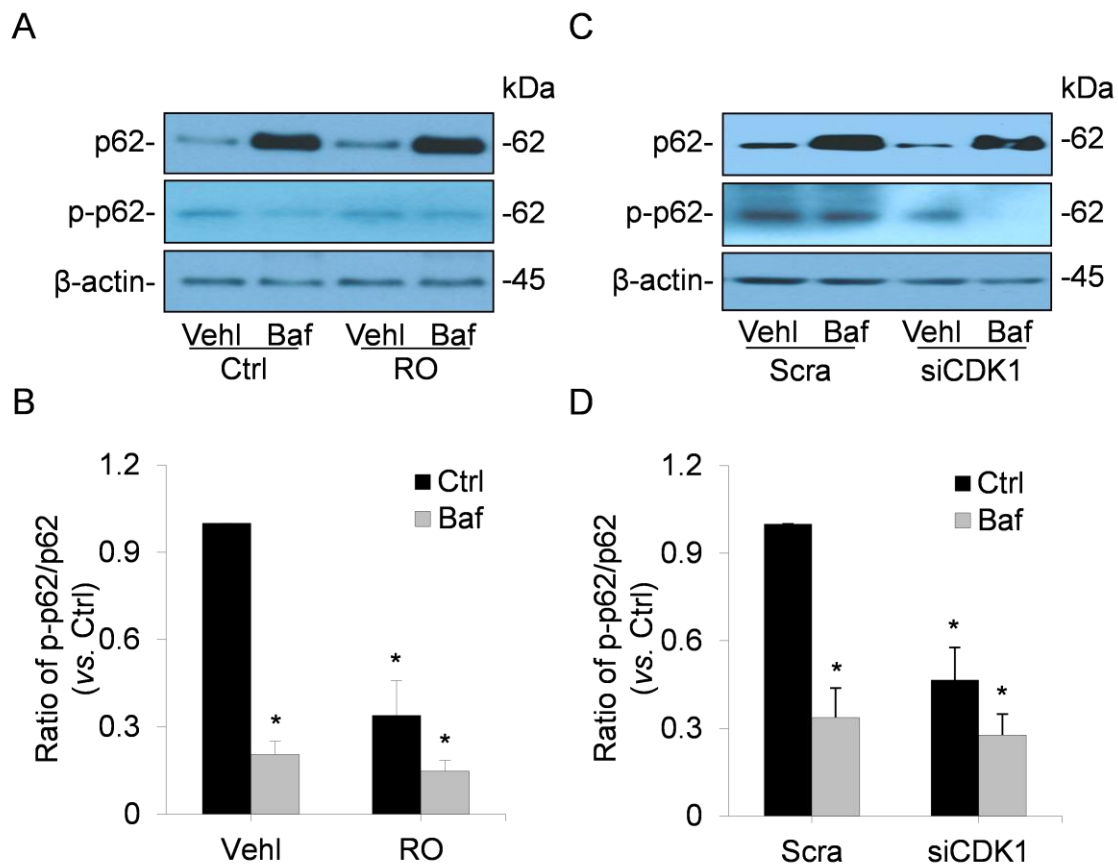


Figure 8. Reduction of p62 phosphorylation during inhibition of lysosome function. A. Representative Western blot images showing the expression of p62 and p-p62 in different groups. B. Summarized data showing expression of p62 and p-p62, quantitated as a ratio of p-p62 band over p62 band (n=5). C. Representative gel documents showing the expression of p62 and p-p62 in different groups. D. Summarized data showing expression of p62 and p-p62, quantitated as a ratio of p-p62 band over p62 band (n=4). * P<0.05 vs. Ctrl.

CHAPTER SEVEN

DISCUSSION

The major goals of the present study were to determine whether lysosome dysfunction-enhanced podocyte EMT is attributable to the accumulation of autophagosomes and p62, which are degraded during the normal autophagic process, and to explore the mechanisms by which p62 exerts its action as a signaling hub to activate or enhance EMT in podocytes. It was found that inhibition of lysosome function using a low dose of lysosomal V-ATPase inhibitor, Baf A1 (5 nM) or by silencing the V-ATPase gene resulted in a marked enhancement of podocyte EMT, an effect that was accompanied by autophagosome accumulation due to the deficient autophagic flux. This enhancement of EMT in podocytes was significantly attenuated by inhibiting autophagosome formation with Sp-1, suggesting that autophagosome accumulation in podocytes is required for enhancement of EMT by lysosome dysfunction. We further explored the signaling mechanism(s) responsible for this effect. Given that p62 was found to be significantly increased during lysosome dysfunction and resulting deficiency of autophagic flux, several signaling pathways known to regulate p62 were investigated including Nrf2-mediated redox signaling, NF- κ B-dependent transcriptional regulation, and CDK1-mediated phosphorylation of p62 as an intracellular controller of cell mitosis exit. Our results indicate that reduction of CDK1-mediated p62 phosphorylation may contribute to the enhancement of EMT by lysosome dysfunction.

It has been reported that podocytes in glomeruli of mammalian animals are highly differentiated and therefore the long-term survival and maintenance of their structural and functional integrity are greatly dependent upon the autophagic process [12, 18]. This led us to hypothesize that

lysosome dysfunction and consequent derangement of autophagic flux may be an important mechanism activating or enhancing EMT in podocytes, leading to podocyte injury and glomerular sclerosis. This hypothesis was tested in the present study by inhibiting lysosome function using the V-ATPase inhibitor, Baf A1, and a specific V-ATPase siRNA. We indeed demonstrated that inhibition of lysosome function by Baf A1 and V-ATPase siRNA significantly enhanced EMT in podocytes, which was comparable to that produced by a well-established EMT inducer, TGF- β [19, 20] or by deletion or knocking down of CD38, an enzyme for production of an endogenous regulator of lysosome function, NAADP [12, 17]. To our knowledge, these results represent the first experimental evidence that inhibition of lysosomal V-ATPase activity or its gene silencing activated or enhanced podocyte EMT. In previous studies, various molecular mechanisms have been proposed to activate EMT in other cell or tissue types, which are similar to those regulating oncogenic properties in neoplastic cells such as proliferation, resistance to apoptosis and angiogenesis through transcription factors [21]. However, these mechanisms are found mainly related to ubiquitination, namely, the covalent link of the small 76-amino acid protein ubiquitin to target proteins, signaling for the proteins to be degraded by the 26S proteasome complex [21]. Although lysosomal degradation of some signaling proteins may be one of mechanisms responsible for EMT, little is known as yet how lysosome dysfunction induces EMT in many cell types including podocytes. In this regard, there is evidence that Raf and TGF- β may work together to promote the lysosomal degradation rather than recycling of E-cadherin in tumor cells, resulting in EMT and tumor progression [22]. Despite these observations of lysosomal degradation of signaling proteins in EMT, it remains unknown how lysosomal degradation of signaling proteins activates EMT. In particular, it is interesting to know whether the reversal of differentiation induced by autophagy, a process fine

controlled by lysosome function, contributes to EMT in podocytes. If so, we need to test what is the triggering mechanism.

We first examined whether enhanced EMT during lysosome dysfunction is associated with deficient autophagy, in particular, the autophagic flux, given the important role of lysosomes in this process. It was demonstrated that autophagosome and p62, a scaffold protein also in autophagic process, were largely accumulated in podocytes by Baf A1 or silencing of v-ATPase gene, suggesting that inhibition of lysosome function leads to abnormal autophagic flux and thereby reduces autophagic degradation of ubiquitinated cargoes and functional substrates involved in the autophagic process such as p62 [23-26]. We also demonstrated that inhibition of autophagosome formation by Sp-1 significantly attenuated the enhancement of EMT induced by lysosome dysfunction induced by Baf A1 and V-ATPase siRNA. To our knowledge, these results provide the first evidence that autophagosome accumulation may serve as a critical mechanism activating or enhancing EMT in podocytes. In some previous studies, autophagy was shown to be critical for the invasion of tumor cells, which is associated with the induction of EMT and activation of TGF- β /Smad3-dependent signaling pathway [27]. In addition, the autophagy process and the autophagy-mediated lysosomal degradation of SNAI/Snail and TWIST, two master inducers of the EMT process have been reported to mediate the effects of death effector domain-containing DNA-binding protein (DEDD) to alter tumor growth and metastasis, suggesting that autophagy is involved in EMT and subsequent growth or metastasis of tumors [28, 29].

After confirmation of deficient autophagic flux as an important process, we went on to address how Baf A1-induced accumulation of autophagosome-initiated EMT in podocytes. It has been reported that p62 as a signaling hub regulates cell proliferation and many other activities [30, 31] and that in tumor cells, p62 is required for cell transformation [30, 32, 33]. Interestingly, the genetic inactivation of key autophagy molecules, such as Atg7, results in p62 accumulation and hepatotoxicity, which leads to the generation of liver tumors [18, 30, 34]. This suggests an important association between p62 and the autophagic process with tumorigenesis or cell transdifferentiation. We tested the role of three pathways regulating p62 function in podocyte EMT, which include NF- κ B, Nrf2 and cyclin-dependent kinases (CDKs). Using selective chemical or genetic inhibitors, we found that inhibition of the NF- κ B or Nrf2 signaling pathways had no effect on Baf A1-induced EMT in podocytes, suggesting that neither pathway is involved in the podocyte transdifferentiation induced by lysosome dysfunction. However, inhibition of CDK1 activity or CDK1 gene silencing produced podocyte EMT without affecting the level of p62. In the presence of CDK1 inhibitor or siRNA, Baf-induced podocyte EMT was markedly attenuated by 60%, which was accompanied by significant reductions in phosphorylation of p62 in podocytes. These results suggest that CDK1 phosphorylation is important for the regulatory control of i podocyte EMT and that reduced CDK1 phosphorylation may result in enhancement of this EMT. To our knowledge, there have been no reports regarding a role of CDK1-mediated p62 phosphorylation in the regulation of podocyte transdifferentiation. Our results provide direct evidence that this CDK1-mediated mechanism critically contributes to EMT activation. In studies using other cell types, CDKs were demonstrated to regulate the progression of mammalian cells through the various phases of the cell cycle [35]. Among these CDKs, CDK1 controls transit through the late S/G2 phase and early mitosis phase of the cell cycle [36, 37],

which may be associated with p62 phosphorylation at residues T269 and S272 [38, 39]. In cancer cells, expression of a nonphosphorylatable p62 mutant displayed higher tumorigenic properties than the same cells expressing wild-type p62 [39]. Moreover, p62 phosphorylation has been shown to play an important role in the stabilization of cyclin B1, which interacts with CDK1 to specifically regulate the entry into mitosis [39]. Our findings together with these previous results provide strong evidence that reduced phosphorylation of p62 due to inhibition of CDK1 during lysosome dysfunction leads to a faster exit from cell mitosis and thereby enhances EMT in podocytes, which results from accumulation of p62 induced by deranged autophagic flux.

In summary, the present study revealed a new triggering mechanism of podocyte EMT under conditions of inhibition of lysosome function, which is characterized by deranged autophagic flux, p62 accumulation and associated reduction of p62 phosphorylation. This dysregulation of p62 and its CDK1-dependent phosphorylation may represent a novel early event leading to podocyte dysfunction and injury, which may initiate podocytes injury and ultimately result in glomerulosclerosis during lysosome dysfunction. These results may direct toward the development of new therapeutic strategies targeting phosphorylation of p62 for prevention or treatment of glomerular sclerosis associated with lysosome dysfunction and deficient autophagy under different pathological conditions such as hypercholesterimia, hyperhomocysteinemia or diabetes mellitus.

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