National Cholesterol Awareness Month

Protective Role of Clusterin/Apolipoprotein J Against Neointimal Hyperplasia via Antiproliferative Effect on Vascular Smooth Muscle Cells and Cytoprotective Effect on Endothelial Cells

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Objective—Clusterin is induced in vascular smooth muscle cells (VSMCs) during atherosclerosis and injury-induced neointimal hyperplasia. However, its functional roles in VSMCs and endothelial cells remain controversial and elusive. This study was undertaken to clarify the role of clusterin in neointimal hyperplasia and elucidate its mechanism of action.

Methods and Results—Adenovirus-mediated overexpression of clusterin (Ad-Clu) repressed TNF-α–stimulated expression of MCP-1, fractalkine, ICAM-1, VCAM-1, and MMP-9, leading to inhibition of VSMC migration. Both Ad-Clu and secreted clusterin suppressed VSMC proliferation by inhibiting DNA synthesis, but not by inducing apoptosis. Ad-Clu upregulated p53 and p21$^{\text{cip1/waf1}}$ but downregulated cyclins D and E, leading to suppression of pRb phosphorylation and subsequent induction of G1 arrest in VSMCs. Clusterin deficiency augmented VSMC proliferation in vitro and accelerated neointimal hyperplasia in vivo, but concomitantly impaired reendothelialization in wire-injured murine femoral arteries. Moreover, Ad-Clu significantly reduced neointimal thickening in balloon-injured rat carotid arteries. Clusterin also diminished TNF-α–induced apoptosis of human umbilical vein endothelial cells and restored endothelial nitric oxide synthase expression suppressed by TNF-α.

Conclusion—These results suggest that upregulation of clusterin during vascular injury may be a protective response against, rather than a causative response to, the development of neointimal hyperplasia. (***Arterioscler Thromb Vasc Biol***. 2009;29:1558-1564.)

Key Words: clusterin ■ VSMC ■ endothelial cells ■ proliferation ■ neointimal hyperplasia

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The excessive proliferation and migration of VSMCs from arterial media to intima are the major cause of neointimal hyperplasia, which contributes to the development and progression of vascular pathologies such as atherosclerosis, restenosis after angioplasty, and vein-graft failure.[1–5] Cytokines and growth factors such as TNF-α and PDGF participate in these processes. In such pathological conditions, VSMCs express several proinflammatory chemokines, cell adhesion molecules, and matrix degrading endopeptidases (MMPs), such as fractalkine, monocyte chemotatic protein (MCP-1), intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and matrix metalloproteinase-9 (MMP-9).[6–10] These molecules are the therapeutic targets for the prevention and treatment of atherosclerosis and postangioplasty restenosis.[11]

Growth factors and cytokines stimulate VSMC proliferation through acceleration of cell cycle progression, which is tightly controlled by several cell cycle–regulatory proteins.[3,5,12] G0/G1 to S-phase transition is triggered by phosphorylation of a key cell cycle regulator, retinoblastoma protein (pRb), resulting in upregulation of genes required for DNA synthesis and cell cycle progression.[13] pRb phosphorylation is mediated by cyclin–cdk complex and thus its phosphorylation status is regulated by the expression levels of both cell cycle–stimulatory (ie, cyclins D, E, and A) and inhibitory proteins (ie, p53 and cdk inhibitors,
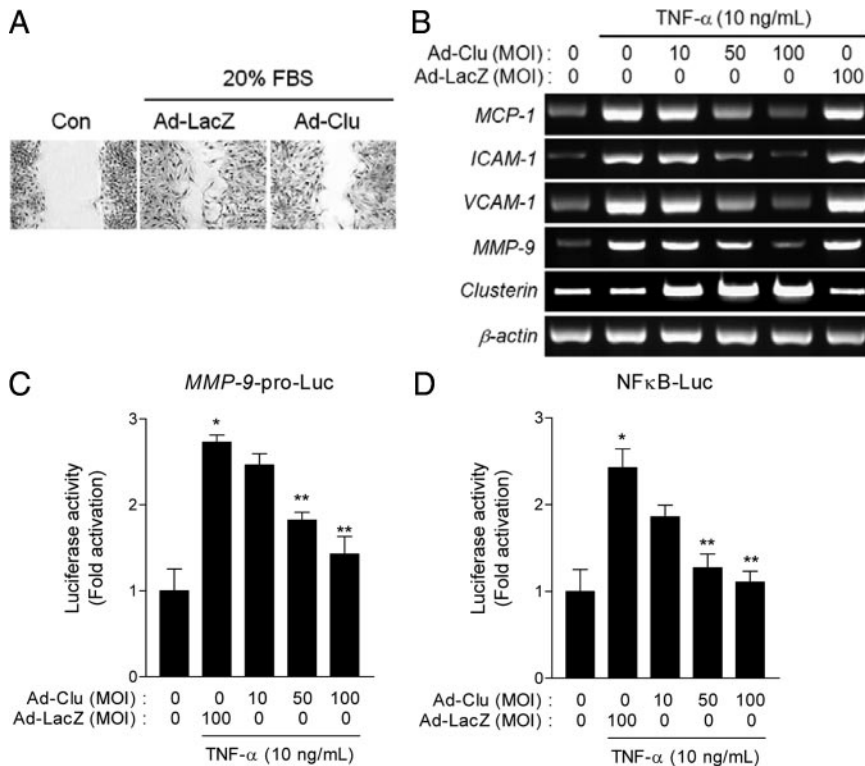
Figure 1. Clusterin inhibits VSMC migration through downregulation of chemokines, cell adhesion molecules, and MMP-9. A, Wound healing assay. VSMCs were infected with Ad-Clu or Ad-LacZ and serum starved for 24 hours. Cells were wounded by razor scraping and then incubated with serum for 24 hours. B, VSMCs infected with Ad-Clu or Ad-LacZ were serum starved for 24 hours and treated with TNF-α for 3 hours or 24 hours (MMP-9). Total RNA was isolated for semiquantitative RT-PCR analysis. C and D, Cos-1 cells infected with Ad-LacZ or Ad-Clu were transfected with MMP-9–pro-Luc (C) and pNF-κB–Luc (D) and stimulated with TNF-α for 24 hours. *$P<0.05$ vs reporter alone, **$P<0.05$ vs TNF-α stimulation. Cells were harvested for luciferase and β-galactosidase assays. Luciferase activity was normalized to β-galactosidase activity and data are expressed as fold transactivation relative to the luciferase activity of the reporter alone. *$P<0.005$ vs reporter alone.

p21[waf1/cip1] and p27[kip1] [hereafter termed p21 and p27]). p53 upregulates p21, and p21 inhibits cyclin–cdk complexes and subsequent pRB phosphorylation, leading to the G1 arrest.

Clusterin (Clu)/Apolipoprotein (Apo) J is a secreted heterodimeric glycoprotein, composed of α and β subunits.[14,15] Clusterin is detected in the intima and media of aortas with atherosclerotic lesions but not in normal aorta, and induced during the progression of atherosclerosis and postangioplasty restenosis.[16–20] Although these data suggest that clusterin plays a crucial role in atherosclerosis and restenosis, its role is still controversial. Some studies have suggested that clusterin protects against atherosclerosis through its antiinflammatory property.[16,18,19] There are also conflicting reports showing that clusterin stimulates or inhibits VSMC proliferation and migration.[20,21] Additionally, endothelial dysfunction and impaired reendothelialization play an important role in the development of atherosclerosis and restenosis.[22–24] However, little study has been performed to clarify the role of clusterin in endothelial cell (EC) apoptosis or dysfunction. Therefore, it is still unclear whether clusterin promotes or prevents neointimal hyperplasia, and its detailed molecular mechanism of action remains to be elucidated.

Here, we demonstrate that clusterin deficiency exacerbates neointimal hyperplasia and impairs reendothelialization in wire-injured femoral arteries. We also elucidate the molecular mechanism by which clusterin suppresses VSMC proliferation and exerts cytoprotective effects on ECs. Therefore, our data suggest that clusterin plays a critical role in protection against restenosis after vascular injury.

Materials and Methods

Animals
Four-week-old male Sprague-Dawley (SD) rats (Hyochang, Daegu, Korea) were used in the experiments. To generate both WT and Clu KO mice with the genetic background of C57BL/6J, clusterin deficient mice originally generated using a *Swiss black* genetic background[25] were backcrossed to C57BL/6J strains for at least 7 generations. All procedures complied with the institutional guidelines for animal research.

For expanded Materials and Methods, please see the supplemental materials (available online at http://atvb.ahajournals.org).

Results

Clusterin Inhibits VSMC Migration by Inhibiting TNF-α/NF-κB Signaling Pathway

To clarify the role of clusterin in the VSMC migration and proliferation, we used adenovirus expressing a secreted form of clusterin (Ad-Clu) or LacZ (Ad-LacZ). Clusterin expression was confirmed by Western blot analysis (supplemental Figure I). To investigate the effect of clusterin on VSMC migration, wound-healing assay was performed in VSMCs infected with Ad-Clu or Ad-LacZ. Serum-stimulated migration of VSMCs was significantly inhibited by clusterin overexpression (Figure 1A).

To explore the molecular mechanism of antimigratory action of clusterin, we examined the expression of chemokines, cell adhesion molecules, and MMP-9, which play important roles in VSMC migration and proliferation.[6–10] Semiquantitative RT-PCR analysis showed that Ad-Clu markedly decreased TNF-α–stimulated mRNA expression of MCP-1, ICAM-1, VCAM-1, and MMP-9 in a multiplicity of infection (MOI)-dependent manner (Figure 1B). Protein levels of fractalkine and VCAM-1 were significantly diminished by Ad-Clu (supplemental Figure I). Additionally, clusterin repressed the TNF-α–stimulated promoter activity of MMP-9 (Figure 1C). Because all these molecules are upregulated by TNF-α/NF-κB signaling pathway and contribute to VSMC migration and proliferation,[6–11] we examined whether clusterin could affect the TNF-α–stimulated NF-κB signaling.
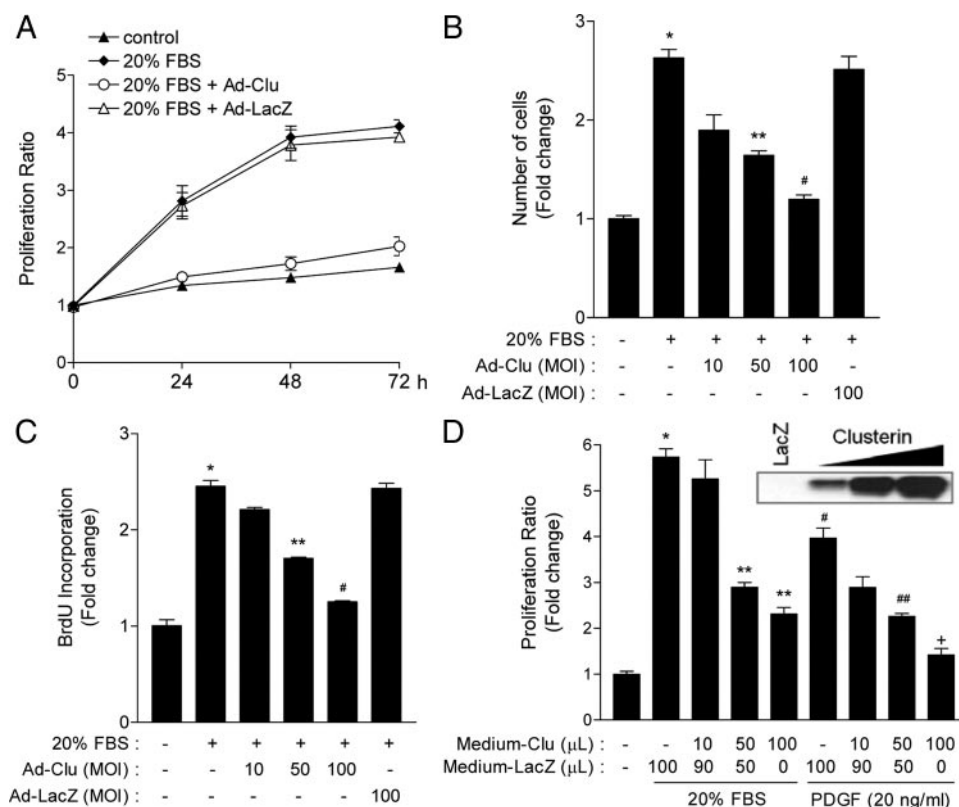
Figure 2. Clusterin suppresses VSMC proliferation through inhibition of DNA synthesis. VSMCs were infected with Ad-LacZ or Ad-Clu, serum starved for 48 hours, and stimulated with serum for 24, 48, and 72 hours (A, B) or 24 hours (C). The proliferation rate of VSMCs was determined by WST-1 cell proliferation assay (A, D), trypan blue cell counting (B), and BrdU incorporation assays (C). B and C, *$P<0.01$ vs control, **$P<0.05$, #$P<0.01$ vs serum stimulation. D, VSMCs were serum starved for 48 hours and incubated with conditioned media (Medium-Clu or Medium-LacZ) together with serum or PDGF-BB for 72 hours. *$P<0.005$, #$P<0.01$ vs control, **$P<0.01$ vs serum stimulation, ##$P<0.05$, +$P<0.01$ vs PDGF stimulation. Clusterin level in conditioned medium used in this assay was confirmed by Western blotting.

Clusterin repressed TNF-α–induced transactivity of NF-κB (Figure 1D), whereas it had little effect on Egr-1–, CREB-, and AP-1–dependent reporter activities (supplemental Figure II), suggesting its specificity for NF-κB inhibition. These results suggest that clusterin inhibits VSMC migration by downregulating chemokines, cell adhesion molecules, and MMP-9, at least in part, through inhibition of NF-κB activity.

Clusterin Reduces VSMC Proliferation by Inhibiting DNA Synthesis

To investigate the effect of clusterin on VSMC proliferation, WST-1 cell proliferation and trypan blue cell counting assays were performed. Serum treatment stimulated proliferation of quiescent VSMCs, and this serum-stimulated VSMC proliferation was significantly diminished by Ad-Clu but not by Ad-LacZ (Figure 2A). Trypan blue cell counting assay also showed that Ad-Clu reduced serum-stimulated increase in cell numbers (Figure 2B). Because WST-1 and cell counting assays reflect not only cell proliferation but also viability and cytotoxicity, BrdU incorporation assay was performed to investigate the effect of clusterin on the DNA synthesis. Similar to the results from WST-1 and cell counting assays, Ad-Clu strongly inhibited DNA synthesis induced by serum stimulation (Figure 2C).

To exclude the possibility that excessive secretion of clusterin by Ad-Clu may influence VSMC proliferation, the direct effect of secreted form of clusterin was examined. Incubation of VSMCs with conditioned medium containing secreted clusterin, but not control medium resulted in a strong inhibition of both serum- and PDGF-stimulated VSMC proliferation (Figure 2D). These observations suggest that both clusterin overexpression and secreted clusterin have an antiproliferative effect on VSMCs through inhibition of DNA synthesis.

Clusterin Induces G1 Phase Cell Cycle Arrest by Inhibiting pRb Phosphorylation

To further analyze which phase of cell cycle is controlled by clusterin and whether apoptosis is involved in antiproliferative effect of clusterin, we used a FACS cell cycle analysis. Serum stimulation of VSMCs synchronized in G0/G1 phase dramatically increased the percentage of cells in S phase (Figure 3A). Ad-Clu, but not Ad-LacZ, strongly reduced this serum-stimulated accumulation of cells in S phase and concomitantly increased cell numbers in G0/G1 phase, consistent with the result from Figure 2C. Notably, Ad-Clu did not induce any significant accumulation of cells in Sub G1 phase, indicating that apoptosis is not involved in the antiproliferative role of clusterin in VSMCs.

Next, we investigated whether clusterin affects the phosphorylation of pRb (ppRb) which is required for the cell cycle progression from G0/G1 to S phase.[12,13] Phosphorylated pRb levels were dramatically increased from 6 to 24 hours after serum stimulation, whereas total pRb proteins were decreased (Figure 3B). This is probably because phosphorylated forms of pRb are subject to ubiquitination and degradation. Ad-Clu significantly diminished phosphorylated pRb and concomitantly increased total pRb in an MOI-dependent manner (Figure 3C). These results propose that clusterin suppresses VSMC proliferation through G1 phase arrest by inhibiting pRb phosphorylation.

Finally, we evaluated the effect of clusterin on the expression of cell cycle–regulatory proteins which regulate pRb phosphorylation status. The expression of cell cycle–promoting proteins (ie, cyclin D and cyclin E) was significantly diminished by Ad-Clu (Figure 3D). In contrast, clusterin
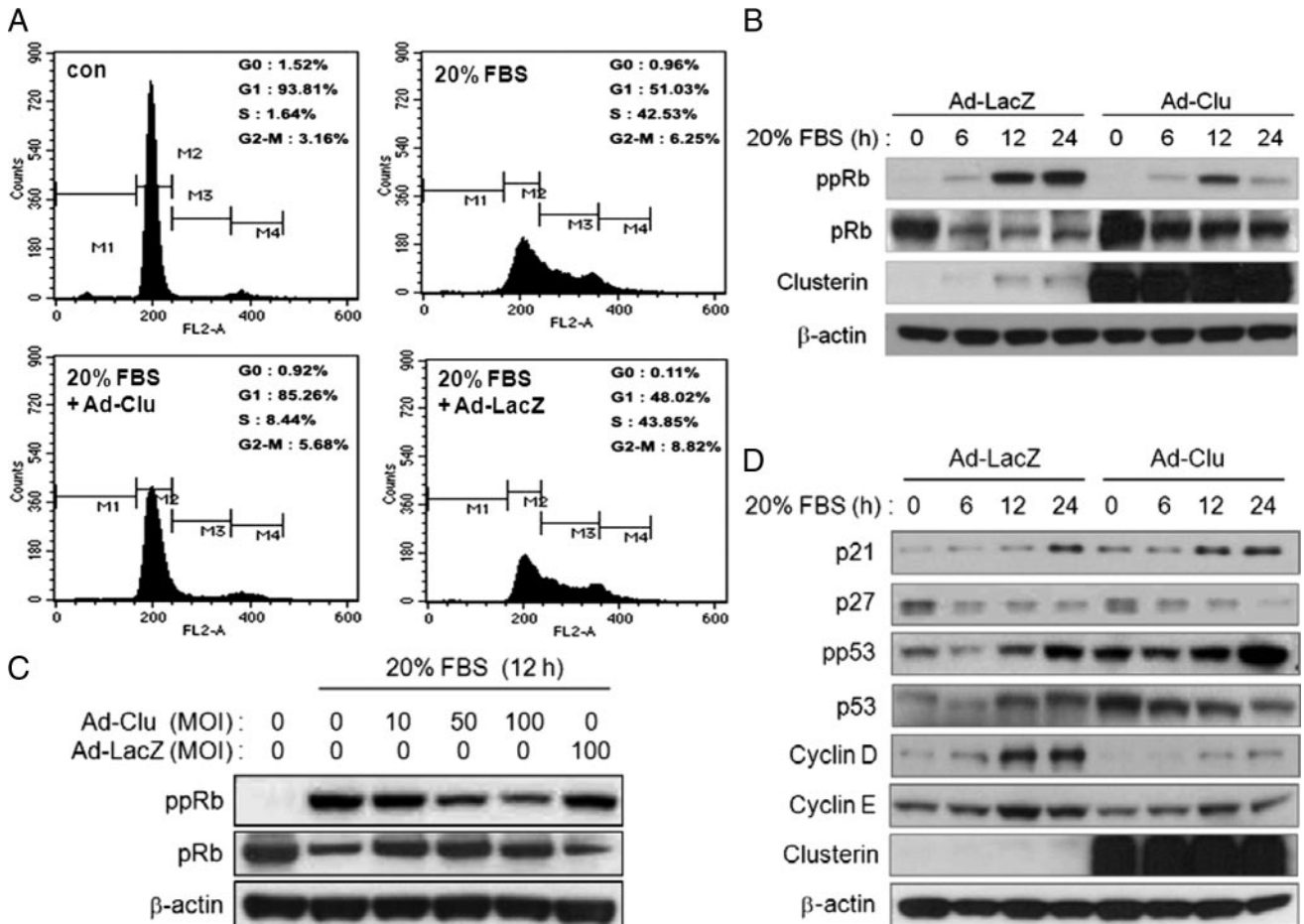
Figure 3. Clusterin induces G1 cell cycle arrest but not apoptosis by inhibiting pRb phosphorylation in VSMCs. A, VSMCs were infected with Ad-LacZ or Ad-Clu and serum starved for 72 hours. Cells were stimulated with serum for 20 hours, and cell cycle was analyzed by FACS. B through D, VSMCs infected with Ad-Clu or Ad-LacZ were serum starved for 24 hours. Cells were stimulated with serum for the indicated time, and Western blot analysis for cell cycle–regulatory proteins was performed.

concomitantly increased key cell cycle–inhibitory proteins including p53, phospho-p53 and p21, but not p27. Additionally, Ad-Clu increased *p53* and *p21* mRNA levels and stimulated the *p53* and *p21* gene promoters (supplemental Figure III), indicating that clusterin regulates p53 and p21 expression at the transcriptional level. These results suggest that clusterin induces G1 phase cell cycle arrest by inhibiting pRb phosphorylation but not by inducing apoptosis, and that this effect is mediated through downregulation of cyclins D and E, and upregulation of p53-p21 pathway.

Clusterin Deficiency Augments VSMC Proliferation

To further confirm the antiproliferative property of endogenous clusterin, we examined the effect of clusterin deficiency on VSMC proliferation using VSMCs obtained from wild-type (WT) and clusterin homozygote knock-out (Clu KO) mice (Figure 4A). Consistent with the results using Ad-Clu (Figure 2), serum-stimulated proliferation of Clu KO VSMCs was significantly enhanced compared with that of WT VSMCs (Figure 4B). This increased proliferation of Clu KO VSMCs was reversed by infection with Ad-Clu, but not with Ad-LacZ (Figure 4C). Consistent with augmented proliferation of Clu KO VSMCs, p21, phospho-p53 and total pRb proteins were reduced, whereas phospho-pRb and cyclins D

and E proteins were upregulated in Clu KO VSMCs (Figure 4D). These results suggest that endogenous clusterin is indispensable for the inhibition of VSMC proliferation.

Essential Role of Clusterin in Protection Against Neointimal Hyperplasia In Vivo

To evaluate the in vivo role of clusterin in neointimal hyperplasia, two vascular injury models (ie, wire- and cuff-injuries) were used in femoral arteries of WT and Clu KO mice. Based on the antiproliferative and antimigratory properties of clusterin, we assumed that neointima formation would be accelerated in Clu KO mice. Thus, we examined the femoral arteries 7 days earlier than usual (ie, 21 or 14 days after wire injury or cuff placement, respectively). At 21 days after wire injury, neointima formation was significantly developed in wire-injured arteries of WT mice, whereas there was no intimal thickening observed in the noninjured control femoral arteries of either WT or Clu KO mice (Figure 5A and 5B). Consistent with the result of augmented proliferation of Clu KO VSMCs (Figure 4), neointimal hyperplasia in wire-injured Clu KO arteries was exacerbated compared with that of WT arteries. Clusterin was expressed in the neointimal region in wire-injured arteries of WT but not Clu KO mice, whereas it was not detectable in either noninjured WT or Clu KO arteries (Figure 5C), in agreement with a previous
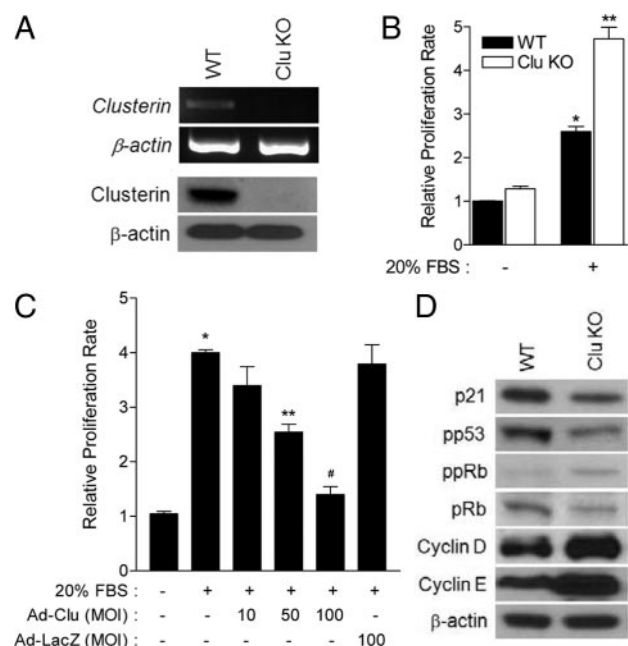
Figure 4. Clusterin deficiency augments VSMC proliferation. A, Semiquantitative RT-PCR (upper) and Western blot (lower) analyses were performed using VSMCs isolated from wild type (WT) or clusterin knockout (Clu KO) mice. B and C, WST assays. VSMCs isolated from WT or Clu KO mice were serum starved and stimulated with serum. *$P<0.05$ vs WT control, **$P<0.05$ vs WT serum stimulation. C, Clu KO VSMCs were infected with 100 MOI of Ad-LacZ or the indicated MOI of Ad-Clu for 2 hours and serum starved for 48 hours. VSMCs were stimulated with serum for 72 hours, and the proliferation rate of VSMCs was determined by WST-1 assay. *$P<0.01$ vs Clu KO control, **$P<0.05$, #$P<0.01$ vs Clu KO serum stimulation. D, WT or Clu KO VSMCs were cultured, and protein levels of cell cycle–regulatory proteins were analyzed by Western blotting.

report.[26] Similarly, neointima formation was accelerated in cuff-injured femoral arteries of Clu KO mice compared with that of WT mice (supplemental Figure IV).

Next, to examine whether overexpression of clusterin suppresses neointimal hyperplasia in vivo, Ad-GFP or Ad-Clu was infused into rat carotid arteries after balloon injury (BI). BI-induced neointima formation was prominent 14 days after BI and slightly pronounced at 28 days after BI. Ad-Clu but not Ad-GFP significantly reduced neointimal thickening at 28 days, as well as 14 days after BI, suggesting that clusterin suppresses, rather than just delays, neointimal hyperplasia (Figure 5D). Clusterin overexpression was confirmed by immunohistochemistry (supplemental Figure V). Collectively, these observations indicate that clusterin plays a crucial role in protection against neointimal hyperplasia in vivo.

Beneficial Effects of Clusterin on ECs

It has been reported that delayed reendothelialization contributes to accelerated neointimal hyperplasia after vascular injury.[22–24] Thus, we examined endothelial regeneration in wire-injured femoral arteries of WT and Clu KO mice. Immunohistochemistry for an endothelial cell specific CD31 antigen revealed that endothelial cell layer lining the luminal wall was markedly restored in wire-injured WT artery (Figure 6A). In contrast, CD31-positive staining in the luminal surface was extremely weak in wire-injured artery of Clu KO mice, implying that clusterin deficiency impairs or delays postinjury reendothelialization accompanied by accelerated neointimal hyperplasia.

To further explore the role of clusterin in endothelial cell apoptosis, WST cell viability and caspase-3 assays were performed. Clusterin significantly inhibited TNF-α–induced apoptosis of human umbilical vein endothelial cells (HUVECs; Figure 6B and 6C). Next, we investigated the effect of clusterin on the expression of endothelial nitric oxide synthase (eNOS), whose decreased expression or activity has been shown to be associated with endothelial dysfunction.[23] TNF-α considerably repressed eNOS expression, and this inhibition was restored by Ad-Clu but not by Ad-LacZ (Figure 6D). Additionally, Ad-Clu reduced TNF-α–induced ICAM-1 and VCAM-1 expression in HUVECs, indicating its antiinflammatory property. Collectively, these findings propose that clusterin may facilitate reendothelialization after vascular injury by exerting cytoprotective effects on ECs.
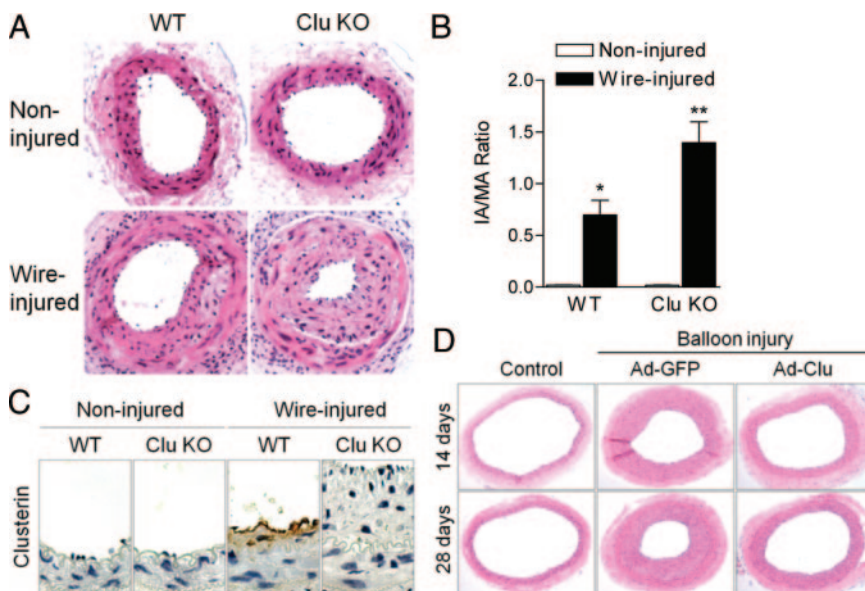

Figure 5. Essential role of clusterin in protection against neointimal hyperplasia in vivo. A, Representative cross-sections of noninjured and wire-injured femoral arteries of wild-type (WT) and Clu KO mice. Left femoral arteries were obtained from WT and Clu KO mice 21 days after wire injury (hematoxylin and eosin stain; original magnification, ×100). B, Neointima formation was presented by the ratio of intimal to medial area (IA/MA). Bars represent the mean±SEM of each of group (n=6); *$P<0.005$ vs noninjured WT, **$P<0.05$ vs wire-injured WT. C, Immunohistochemical staining for clusterin on cross-sections of noninjured and wire-injured femoral arteries of WT and Clu KO mice (original magnification, ×400). D, Representative cross-sections of noninjured and balloon-injured (BI) rat carotid arteries infused with Ad-GFP or Ad-Clu after BI (hematoxylin and eosin stain; original magnification, ×100). Right common carotid arteries were obtained 14 and 28 days after BI.
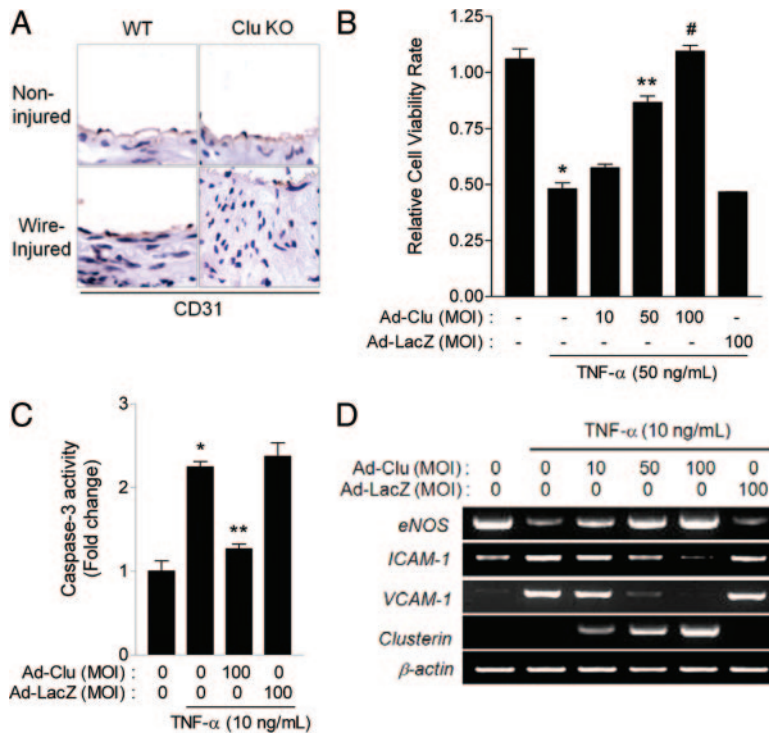
Figure 6. Beneficial Effects of clusterin on endothelial cells. A, To determine of reendothelialization, cross-sections of control and wire-injured femoral arteries of WT and Clu KO mice were subjected to immunohistochemical staining for an endothelial-specific CD31antigen (original magnification, ×400). B and C, Clusterin reduced the TNF-α–induced apoptosis of HUVECs. HUVECs were infected with Ad-LacZ or Ad-Clu and incubated with culture medium containing 1% FBS for 24 hours. Cells were treated with TNF-α for 48 hours, and WST cell viability (B) and caspase-3 assays (C) were performed. B, *$P<0.01$ vs control, **$P<0.05$ and #$P<0.01$ vs TNF-α treatment. C, *$P<0.05$ vs control, **$P<0.05$ vs TNF-α treatment. D, HUVECs infected with Ad-Clu or Ad-LacZ were serum starved for 24 hours, treated with TNF-α for 6 hours, and then total RNA was isolated for semiquantitative RT-PCR analysis.

Discussion

In the present study, we demonstrate that clusterin inhibits VSMC proliferation and migration in vitro and that clusterin deficiency exacerbates and overexpression of clusterin inhibits neointimal hyperplasia induced by vascular injury in vivo. Furthermore, we showed that clusterin has antiapoptotic and antiinflammatory effects on HUVECs, and its deficiency impairs reendothelialization after wire injury in murine femoral arteries in vivo. Therefore, protective role of clusterin against neointimal hyperplasia may be owing to antiproliferative and antimigratory effects on VSMCs and cytoprotective effect on ECs (supplemental Figure VI).

Clusterin reduced the TNF-α–stimulated expression of chemokines, cell adhesion molecules, and MMP-9 by repressing NF-κB in VSMCs. The TNF-α/NF-κB signaling pathway has also been shown to play an important role in endothelial cell apoptosis and dysfunction by downregulating eNOS and upregulating ICAM-1 and VCAM-1.[27,28] Therefore, our results suggest that clusterin may regulate function of VSMCs and ECs through repression of TNF-α/NF-κB signaling pathway. Moreover, differential effect of clusterin on VSMCs and ECs may be, at least in part, attributable to the intrinsically distinct effect of TNF-α on these cells, namely proliferative and promigratory effect on VSMCs and proapoptotic effect on ECs.[6–11,26,27] Clusterin also downregulated cyclins D and E, whereas it upregulated p53 and p21, leading to inhibition of pRb phosphorylation and resultant DNA synthesis in VSMCs. The expression and phosphorylation of these proteins were inversely altered in clusterin KO VSMCs. Therefore, our findings suggest that clusterin inhibits VSMC proliferation via induction of G1 cell cycle arrest accompanied by reduced pRb phosphorylation by downregulating cell cycle–promoting factors and upregulating p53-p21 pathway.

However, the molecular mechanism by which clusterin upregulates *p53* gene expression awaits further investigations.

As aforementioned, previous studies dealing with VSMC proliferation and migration reported quite conflicting results.[20,21] Thus, the meaning of clusterin induction during development of neointimal hyperplasia has remained to be defined. To properly address this matter, loss of function study using Clu KO mice would provide more useful information. Here, we demonstrate that clusterin deficiency increases VSMC proliferation in vitro and exacerbates neointimal hyperplasia in vivo. Additionally, re-endothelialization was impaired in wire-injured Clu KO but not WT arteries. However, Ad-Clu did not affect either migration or proliferation of HUVECs (supplemental Figure VII). Instead, clusterin inhibited TNF-α–induced apoptosis of HUVECs, whereas it restored the eNOS expression which was repressed by TNF-α. These results suggest that clusterin might facilitate reendothelialization not through promoting migration and proliferation of neighboring endothelial cells, but through exerting cytoprotective and antiinflammatory effects on endothelial cells adjacent to the injury sites, where TNF-α is highly increased. It is also possible that clusterin may promote mobilization or homing of circulating endothelial progenitor cells which contribute to endothelial regeneration, thereby limiting neointimal hyperplasia.[24] Further studies using bone marrow transplantation between WT and Clu KO are necessary to verify this possibility. Collectively, these results suggest that clusterin exerts beneficial effects on the vascular wall at the level of both endothelial cells and VSMCs, resulting in protection against neointimal hyperplasia.

Clusterin is a complex molecule and it is not easy to identify its physiological role in vivo, because there are at least 2 isoforms including secreted (sClu) and nuclear forms (nClu) of clusterin and they show quite different roles in various cancers.[29] However, function of sClu as a tumor promoter or a suppressor

is still debated depending on type and stage of cancers, although nClu is generally considered to be proapoptotic.[30] Additionally, a previous study showed that upregulation of nClu and downregulation of sClu by all-*trans* retinoic acid (RA) mediate proapoptotic and antiproliferative effect of RA on VSMCs, respectively.[26] However, they did not directly demonstrate distinctive role of nClu and sClu on VSMC apoptosis and proliferation through overexpression system either in vitro or in vivo. Hence, the differential effect of sClu and nClu on VSMCs and ECs is still unclear. Here, we demonstrated that conditioned medium containing sClu as well as adenovirus expressing sClu (Ad-Clu) inhibited VSMC proliferation and migration (supplemental Figure VIII) and Ad-Clu has antiapoptotic effect on ECs. Notably, sClu was internalized into VSMCs 2 hours after treatment of the conditioned medium containing sClu (supplemental Figure IX). Therefore, we suggest that clusterin primarily as a secreted form exerts both antiproliferative effects on VSMCs and prosurvival on ECs. Moreover, Ad-Clu extensively suppressed neointimal thickening in balloon-injured rat carotid arteries. These results demonstrate that sClu inhibits neointimal hyperplasia in vivo. However, further studies are necessary to verify the in vivo and in vitro roles of nClu in neointimal hyperplasia as well as in VSMCs and ECs. From these findings, we propose that clusterin stimulators or small peptides mimicking the sClu action might be used locally or regionally, but not systematically in drug-eluting stents to prevent postangioplasty restenosis, while minimizing the adverse effects, if any, of clusterin such as carcinogenic effect.

In summary, our present study provides new insights into the role of clusterin during vascular injury. Clusterin induction may be a necessary and compensatory response to prevent neointimal hyperplasia in acute and chronic vascular injuries including balloon angioplasty and atherosclerosis.

Sources of Funding

Disclosures

None.

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Arteriosclerosis, Thrombosis, and Vascular Biology

Protective Role of Clusterin/Apolipoprotein J Against Neointimal Hyperplasia via Antiproliferative Effect on Vascular Smooth Muscle Cells and Cytoprotective Effect on Endothelial Cells

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Supplement Material

Materials and Methods

Cuff- or wire-induced murine vascular injury models, and histological and morphometric analyses.

Vascular injuries (cuff- or wire-injury) were carried out in 20-week-old male WT and Clu KO mice by placing a nonconstricting cuff loosely around femoral arteries or inserting a straight spring guidewire into femoral arteries. At 14 days after cuff placement or 21 days after wire injury, femoral arteries were harvested, fixed overnight in 4% formaldehyde in PBS and paraffin-embedded. Serial cross sections (4 μm thick) of femoral arteries were stained with hematoxylin and eosin (H&E) and used for histological and morphometric analyses. For morphometric analysis, intimal and medial areas were measured with a digitizing system (model INTUOS 6.8, Wacom, Vancouver, WA) and analyzed using image analysis software (Scion Co., Frederick, MA).

Balloon-injury (BI) rat carotid artery and delivery of adenoviruses into injured arteries

Rat carotid artery balloon injury using 7-week-old male Sprague-Dawley (SD) rats was performed as described previously (1). To deliver adenoviruses expressing clusterin (Ad-Clu) or Ad-GFP after balloon injury, 100 μl of Ad-GFP (10^{10} pfu/ml) or Ad-Clusterin (10^{10} pfu/ml) were infused into the ligated segment of the common carotid artery for 30 minutes. Viral transductions were evaluated at 2 and 14 days after balloon injury by isolation of arteries followed by either examining cryosections for green fluorescence protein (GFP) expression at 2 days after BI or by immunohistochemical staining of clusterin at 14 days after

BI. At 14 days and 28 days after BI, right and left common carotid arteries were harvested, fixed overnight in 4% formaldehyde in PBS and paraffin-embedded. Serial cross sections (4 μm thick) of femoral arteries were stained with hematoxylin and eosin (H&E) and used for histological and morphometric analyses.

Plasmids

The -1,895/+281 *p53*-Luc was constructed by insertion of PCR product of the human *p53* promoter into *Kpn I/Bgl II* sites of pGL3 basic vector. PCR primers used are as follows; forward, 5'-CGGGGTACCGAATCCTTGAGGGAAGTA-3' and reverse 5'-GGAAGATCTTA CACGGAGCCGAGAGCC-3'. Reporter plasmids, -2300/+8 *p21*-Luc (2), pNF-κB-Luc (3), *MMP-9*-pro-Luc (3), CRE-Luc (4), AP-1-Luc (5) or pEBS-Luc (6), and pCMV-Egr-1 were described previously.

Cell culture and transient transfection assay.

As previously described (1, 7), VSMCs were isolated from the thoracic aorta of 4-week-old SD rats and 5-week-old male WT and Clu KO mice using the explant and enzyme digestion methods, respectively. VSMCs were cultured in DMEM supplemented with 10% FBS. VSMC purity was determined by positive staining with smooth-muscle-specific α-actin monoclonal antibody (Sigma, St. Louis, MO). Cells from the third to fifth passage were used for the experiments. Human umbilical vein endothelial cells (HUVECs) were cultured in complete endothelial growth medium (EGM)-2 Bullet Kit (Cambrex, Walkersville, MD). HepG2 cells were cultured in MEM, and HEK-293 and Cos-1 cells were cultured in DMEM, supplemented with 10% FBS. For transient transfection assay, HepG2 (8×10^4), Cos-1 (3×10^4) or HEK-293 (3×10^4) cells were seeded in 24-well plate, and infected with 100 MOI of

Ad-LacZ or 10, 50, and 100 MOI of Ad-Clu for 2h. Cells were transfected with 200 ng/well of -1,895/+281 *p53*-Luc, -2300/+8 *p21*-Luc, *MMP-9*-pro-Luc, pNF-κB-Luc, CRE-Luc, AP-1-Luc or pEBS-Luc and 100 ng/well of pCMV-Egr-1 using TransIT-LT1 transfection reagent (Mirus Bio Incorporation, Madison, WI). Cytomegalovirus (CMV)-β-galactosidase plasmids were cotransfected as an internal control. After 48 h, the cells were harvested for luciferase and β-gal assays. Luciferase activity was normalized to the β-galactosidase activity.

Preparation of recombinant adenovirus and conditioned medium (secreted clusterin)

The cDNA encoding rat clusterin was inserted into the *Bgl II/Xho I* sites of the pAd-Track-CMV shuttle vector. To produce the recombinant adenoviral plasmid, the resultant shuttle vector was electroporated into BJ5138 cells containing the AdEasy adenoviral vector. The recombinant adenoviral plasmids were transfected and adenovirus expressing clusterin (Ad-Clu) was amplified in HEK-293 cells, and purified using CsCl density centrifugation (Sigma). The viruses were collected and desalted, and the titers were determined using Adeno-X Rapid titer (BD Bioscience, San Jose, CA). Control adenoviruses (Ad-LacZ and Ad-GFP) were prepared by the same method. To prepare conditioned medium containing secreted clusterin, VSMCs infected with 100 MOI of Ad-LacZ or Ad-Clu for 2 h were incubated with serum free medium for 48 h, and culture (conditioned) medium was collected.

Cell migration assay (Wound healing assay)

VSMCs or HUVECs were seeded on 6-well plates at 90% confluency and infected with 100 MOI of Ad-Clu or Ad-LacZ for 2 h. VSMCs were then serum starved for 24 h and HUVECs were incubated with culture medium containing 1% FBS for 24 h. Cells were wounded by razor scraping, rinsed with PBS, stimulated with medium containing 20% FBS

for 24 h (VSMCs) or 5% FBS for 48 h (HUVECs) and then stained with hematoxylin.

Cell proliferation and BrdU incorporation assays

For WST-1 cell proliferation assay, VSMCs (1.5×10^3) were seeded in 96-well plates, infected with 100 MOI of Ad-LacZ or Ad-Clu for 2 h, and serum starved for 48 h. Cells were then stimulated with 20% FBS for 24, 48, and 72 h. Ten micro litter of working solution containing WST-1 (Dojindo Laboratories, Kumamoto, Japan) was added to each well, and cells were incubated for 2 h at 37°C and the absorbance at 490 nm was measured. For trypan blue cell counting assay, VSMCs (1×10^4) were seeded into 6 well plates and serum starved for 48 h. Cells were stimulated with PDGF-BB (20 ng/ml, Sigma) or FBS (20%) and incubated for 72 hours at 37°C. Cell numbers were determined by trypan blue counting. For 5-bromo-2-deoxy-uridine (BrdU) incorporation assay, VSMCs (1.0×10^3) were seeded into 96-well plates and serum starved for 48 h. Cells were stimulated with 20% FBS for 24 h, and then incubated with 10 μM of BrdU (Roche, Indianapolis, IN) for 6 h. The incorporation of BrdU was determined by the absorbance measured at 450 nm with a reference wavelength at 690 nm using an ELISA plate reader.

FACS analysis

For cell cycle analysis, VSMCs (3×10^5) were seeded into 100 mm culture dish and infected with 100 MOI of Ad-Clu or Ad-LacZ for 2 h. Infected cells were synchronized in G0/G1 phase by serum starvation for 72 h and stimulated with 20% FBS for 20 h. Cells were trypsinized and washed once with cold PBS containing 0.01% $CaCl_2$ and 2% FBS. Cells were fixed in 95% cold ethanol at -20°C for 1 h, stained with 1ml of staining solution (40 μg/ml propidium iodide, 10 μg/ml RNase A and 0.1% NP-40) for 1 h in the dark at 4°C and

analyzed with a FACSCallbur (BD bioscience).

Semi-quantitative RT-PCR

Total RNA was extracted from VSMCs using TRIzol reagent (Invitrogen, Carlsbad, CA) and 2 μg of total RNA was used to synthesize the first strand cDNA using the First Strand cDNA synthesis kit (Fermentas, EU). The first strand cDNAs were amplified by 30 cycles of PCR according to the following PCR parameters: 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, 25 cycles. Primers used in the PCR were as follows: clusterin forward, 5'-CC GGAATTCATGAAGATTCTCCTGCTGTG-3' and reverse, 5'-GCCATCATGGTCTCGTT ACAC-3'; p21 forward, 5'-ATCCTGGTGATGTCCGACCTG-3' and reverse, 5'-TTTCTCT TGCAGAAGACCAAT-3'; p27 forward, 5'-CAGCGCAAGTGGAATTTCGA-3' and reverse, 5'-ATTTTCTTCTGTTCTGTTGGC-3'; p53 forward, 5'-GGCAACTATGGCTTCCACCTG-3' and reverse, 5'-TCCTTCCACCCGGATAAGATG-3'; MCP-1 forward, 5'-ATCA CCAGCAGCAGGTGTCCC-3' and reverse, 5'-CTACAGAAGTGCTTGAGGTGG-3'; rat ICAM-1 forward, 5'-GACCCCAAGGAGATCACATTC-3' and reverse, 5'-AGGAAACA GGCCTTCCAGGGA-3'; human ICAM-1 forward, 5'-ACAGTCACCTATGGCAAC-3' and reverse, 5'-GGTCTGGTTCTTGTGTAT-3'; VCAM-1 forward, 5'-GAACACTCTTACCTGT GCACAGCAAC-3' and reverse, 5'-GGAGCTGGTAGACCCTCGCTGG-3'; eNOS forward, 5'-CTGTGGAAAG ACAAGGCAGC-3' and reverse, 5'-GTCTTCTTCCTGGTGATGCC-3'; MMP-9 forward, 5'-ATTGTCATCCAGTTTGGTGTCGCGGAG-3' and reverse, 5'-AACAG GCTGTACCCTTGGTCTGGA-3'; β-actin forward, 5'-GGCATCGTCACCAACTGGGAC-3' and reverse, 5'-CGATTTCCCGCTCGGCCGTGG-3'.

Western blot analysis

Cell lysates were prepared using a lysis buffer (20 mmol/L Tris, pH7.4, 10 mmol/L Na$_4$P$_2$OH, 100 mmol/L NaF, 2 mmol/L Na$_3$VO$_4$, 5 mmol/l EDTA, pH 8.0, 0.1 mmol/L PMSF, 1% NP-40) containing proteinase and phosphatase inhibitors. Proteins were resolved by SDS-PAGE and transferred to Immobilon-P-membrane (Millipore, Billerica, MA). After blocking, the membrane was incubated with primary antibodies. Anti-clusterin, anti-fractalkine, anti-VCAM-1, anti-pRb, anti-Cyclin D1, and anti-Cyclin E antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho-pRb, anti-p53 and anti-phospho-p53 (ser 15) antibodies from Cell Signaling (Beverly, MA); anti-p21 and anti-p27 antibodies from BD Pharmingen; anti-β-actin antibody from Sigma. The membrane was washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody and signals were detected using the ECL Western blotting detection system (Amersham, Buckinghamshire, UK).

Immunohistochemical staining of clusterin and CD31.

Immunohistochemical staining was performed on the cross sections (4 μm thick) of wire-injured murine femoral arteries using anti-clusterin α antibody (Santa Cruz, SC-6420, 1:50 dilution) or anti-CD31/PECAM-1 antibody (Lab Vision Corporation, MS-1873, 1:50 dilution) and Polink-2 Plus HRP Anti-Goat DAB Detection kit (Golden Bridge International, Inc., Mukilteo, WA) and UltraVision LP Detection System HRP Polymer & DAB Plus Chromogen (Lab Vision Corporation, Fremont, CA) according to the manufacturers' instructions. Cross sections (4 μm thick) of control and balloon-injured rat carotid arteries infused with Ad-GFP or Ad-Clu were subjected to immunohistochemical staining using anti-clusterin antibody (Santa Cruz, SC-6420, 1:50 dilution).

Internalization of secreted clusterin

To verify the internalization of secreted clusterin in the conditioned medium, Western blotting and immunofluorescent staining for clusterin were performed. VSMCs were incubated with conditioned medium (Medium-Clu or Medium-LacZ) for 2 h, then washed with PBS three times and cells were harvested for Western Blotting. For immunofluorescence, VSMCs (2×10^5 cells/well) were seeded, allowed to attach on glass cover slips in 6 well plates for 24 h and pretreated with cycloheximide (CHX, 5 μg/mL) for 2 h to prevent de novo protein synthesis. Cells were incubated with conditioned medium (Medium-Clu or Medium-LacZ) for an additional 2 h, then washed with PBS three times and fixed with 4% paraformaldehyde for 15 min. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature and incubated with anti-clusterin antibody (Santa Cruz, SC-6419, 1:100 dilution) for overnight at 4°C followed by further incubation with Alexa Fluor-568-labeled anti-goat secondary antibody for 3 h at room temperature. Cell nuclei were stained with DAPI (5 μg/mL) and mounted. Samples were analyzed using an inverted MRc5 Carl Zeiss fluorescence microscopy (Thornwood, NY).

HUVEC proliferation and TNF-α-induced apoptosis assays

For WST-1 cell proliferation or viability assay, HUVECs (2×10^4) were seeded in 96-well plates, infected with Ad-LacZ (100 MOI) or Ad-Clu (10, 50 or 100 MOI) for 2 h, and incubated with culture medium containing 1% FBS for 24 h. Cells were then stimulated with 5% FBS for 48 h. To examine the effect of clusterin on the TNF-α-induced apoptosis of HUVECs, HUVECs (2×10^4) were seeded in 96-well plates, infected with Ad-LacZ (100 MOI) or Ad-Clu (10, 50 or 100 MOI) for 2 h, and incubated with culture medium containing 1% FBS for 24 h. Cells were then treated with TNF-α (50 ng/mL) for 48 h. Ten micro litter of

working solution containing WST-1 (Dojindo Laboratories, Kumamoto, Japan) was added to each well, and cells were incubated for 2 h at 37°C and the absorbance at 490 nm was measured. For DEVDase activity (Casepase-3 activity) assay, Enzymatic activities of DEVDase were determined by incubating cell lysates in 100 μl reaction buffer (1% NP-40, 20 mmol/L Tris–HCl, pH 7.5, 137 mmol/L NaCl, and 10% glycerol) containing 5 μM of chromogen substrate [Asp-Glu-Val-Asp-p-nitroanilide (DEVD-pNA)] (Sigma) at 37°C for 2 h. The release of chromophore p-nitroanilide (pNA) was monitored spectrophotometrically (405 nm).

Statistical analysis

Results are expressed as the mean ± S.E.M. Analysis of variance with a subsequent Duncan's test was used to determine significant differences in multiple comparisons. A value of $P < 0.05$ was considered statistically significant. All experiments were performed at least 3 times.

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Supplement Material

Supplemental Figure Legends

Supplemental Figure I. Adenovirus-mediated overexpression of clusterin. A, Rat VSMCs were infected with 50 MOI of Ad-Clu and Ad-LacZ for 24 h and whole cell lysates were subjected to Western blot analysis. B, VSMCs infected with Ad-Clu or Ad-LacZ were serum starved for 24 h, and treated with TNF-α for 3 h. Whole cell extracts were isolated for Western blot analysis.

Supplemental Figure II. **Effect of clusterin on the transcriptional activities of Egr-1, CREB and AP-1.** HEK-293 (3×10^4) cells were seeded in 24-well plate and transfected with 200 ng/well of EBS-Luc together with Egr-1 expression vector (A), CRE-Luc (B), or AP-1-Luc (C). CMV-β-galactosidase plasmids were cotransfected as an internal control. After 24 h, cells were incubated with indicated amount of the conditioned medium (Medium-Clu or Medium-LacZ) together with forskolin (10 ng/mL) or TPA (10 ng/mL for 24 h, and harvested for luciferase and β-gal assays. Luciferase activity was normalized to the β-galactosidase activity and data are expressed as fold transactivation relative to the luciferase activity of the reporter alone. $^*P < 0.05$ versus reporter alone

Supplemental Figure III. Inhibitory effect of clusterin on *p53* and *p21* gene expression. A, VSMCs were infected with Ad-Clu or Ad-LacZ for 2 h, incubated with culture medium for 24 h and harvested to collect total RNA for semi-quantitative RT-PCR analysis. B, HepG2 cells were infected with 100 MOI of Ad-LacZ or 10, 50, and 100 MOI of Ad-Clu for 2 h and

transfected with luciferase reporter plasmids driven by *p53* (B) or *p21* (C) gene promoters for 48 h. Luciferase activity was normalized to β-galactosidase activity and data are expressed as fold transactivation relative to the luciferase activity of the reporter alone. $^*P < 0.05$, $^{**}P < 0.01$ versus reporter alone.

Supplemental Figure IV. **Clusterin deficiency accelerates neointimal hyperplasia in cuff-injured murine femoral arteries.** Representative cross-sections of non-injured and cuff-injured femoral arteries of wild type (WT) and Clu KO mice. Left femoral arteries were obtained from WT and Clu KO mice 14 days after cuff placement (hematoxylin and eosin stain, original magnification, × 200). Neointima formation was presented by the ratio of intimal to medial area (IA/MA). Bars represent the mean ± S.E.M of each of group (n=6); $^*P < 0.01$ versus non-injured WT, $^{**}P < 0.05$ versus cuff-injured WT.

Supplemental Figure V. **Immunohistochemical staining of clusterin in balloon-injured (BI) rat carotid artery.** Ad-GFP or Ad-Clu which also expresses GFP under the separate CMV promoter was infused into the balloon-injured rat carotid arteries. At 2 days and 14 days after balloon injury, rats were sacrificed, and the injured right common carotid arteries and uninjured left common carotid arteries were dissected out; either cryostat or paraffin-embedded cross-sections were made. Cryostat cross sections were fixed and examined for green fluorescence protein expression (top) and paraffin-embedded cross-sections were subjected to immunohistochemical staining using anti-clusterin antibody.

Supplemental Figure VI. Proposed hypothesis illustrating the protective role of clusterin against restenosis after vascular injury.

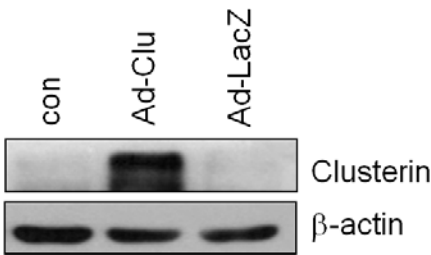**Supplemental Figure VII**. **Effects of clusterin on migration and proliferation of HUVECs.** A. HUVECs were seeded on 6-well plates at 90% confluency and infected with Ad-Clu or Ad-LacZ for 2 h. Cells were incubated with culture medium containing 1% FBS for 24 h, wounded by razor scraping and then stimulated with medium containing 5% FBS for 48 h. B WST cell proliferation assay. HUVECs (2×10^4) were seeded in 96-well plates, infected with indicated MOI of Ad-LacZ or Ad-Clu, and incubated with culture medium containing 1% FBS for 24 h. Cells were then stimulated with 5% FBS for 48 h. $^*P < 0.05$ versus 1% FBS.

Supplemental Figure VIII. Secreted form of clusterin inhibits serum-stimulated VSMC migration. To test the effect of secreted clusterin on VSMC migration, VSMCs were seeded on 6-well plates at 90% confluency and serum starved for 24 h. Cells were wounded by razor scraping and rinsed with PBS. Cells were incubated with conditioned medium (Medium-Clu or Medium-LacZ), together with 20% FBS for 24 h and then stained with hematoxylin.

Supplemental Figure IX. **Secreted clusterin is internalized into VSMCs.** A. VSMCs were incubated with conditioned medium (Medium-Clu or Medium-LacZ) for 2 h, then washed with PBS three times and harvested for Western Blotting. B. VSMCs were seeded and allowed to attach on glass cover slips in 6 well plates for 24 h. Cells were pretreated with cycloheximide (CHX, 5 μg/mL) for 2 h and then incubated with conditioned medium (Medium-Clu or Medium-LacZ) for an additional 2 h. Cells were washed with PBS three times and fixed with 4% paraformaldehyde. Fixed cells were permeabilized and incubated with anti-clusterin antibody followed by incubation with Alexa Fluor-568-labeled anti-goat secondary antibody. Cell nuclei were stained with DAPI.
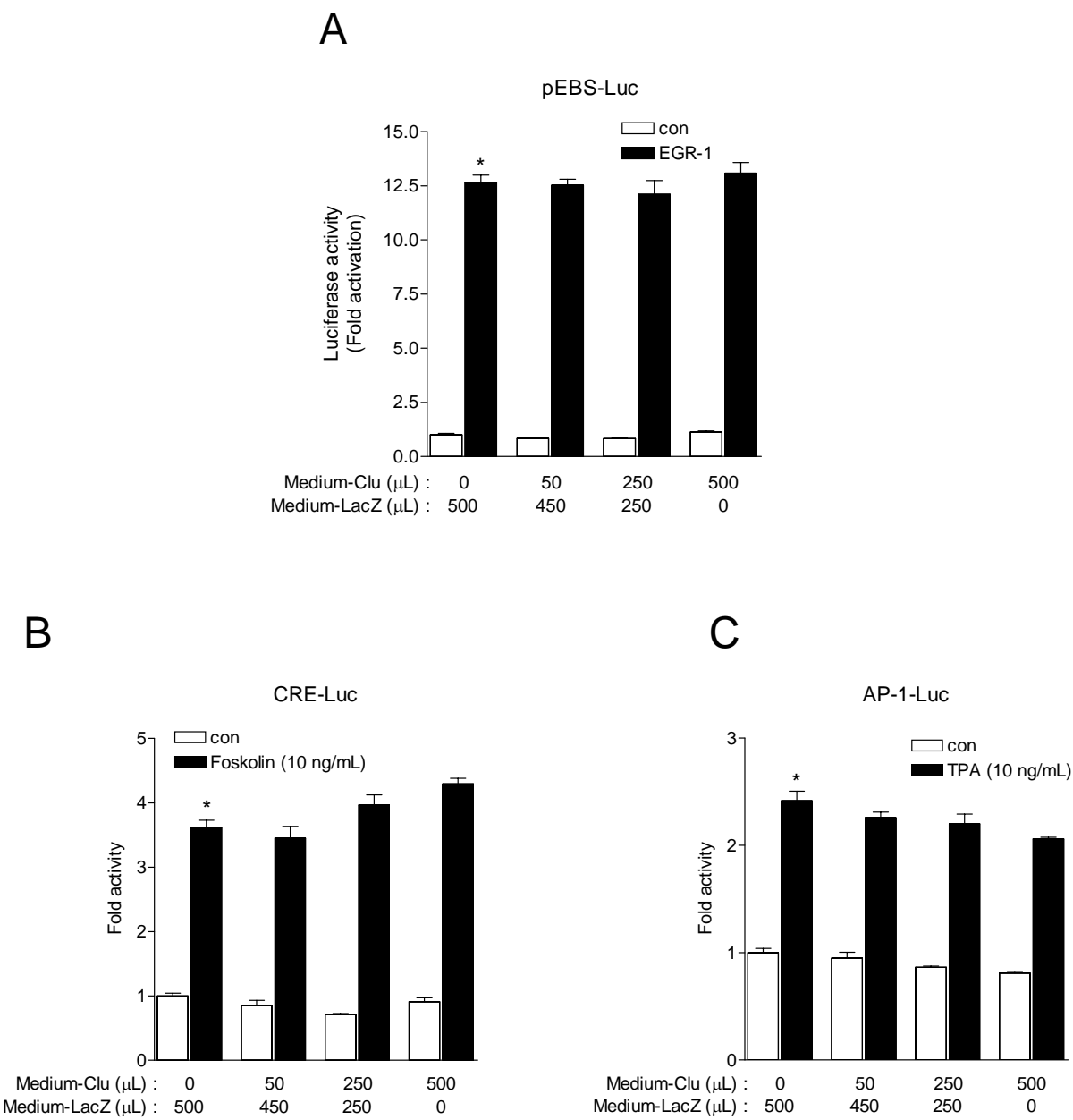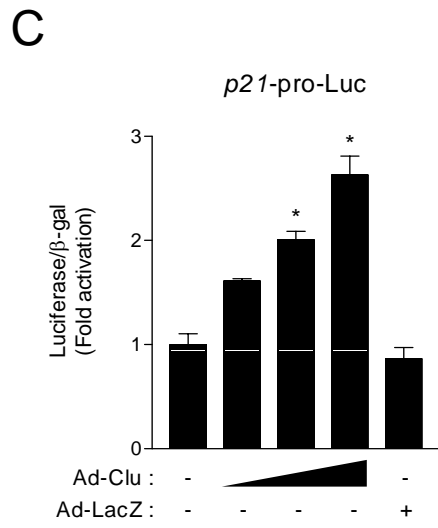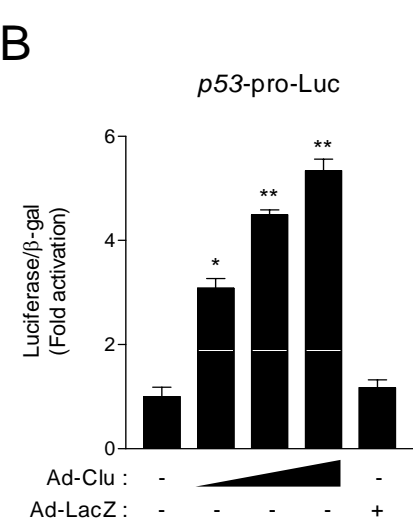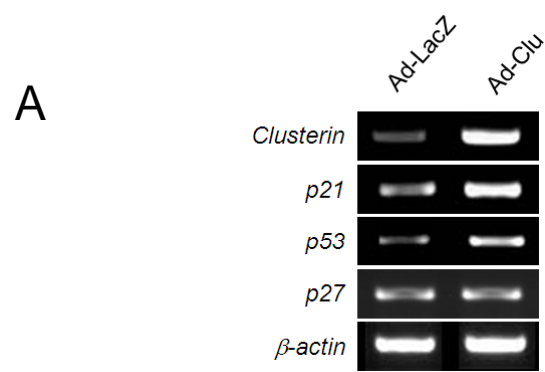
Supplemental Figure I

A

B

Supplemental Figure II

A

pEBS-Luc

B

CRE-Luc

C

AP-1-Luc

Supplemental Figure III

A

B

p53-pro-Luc
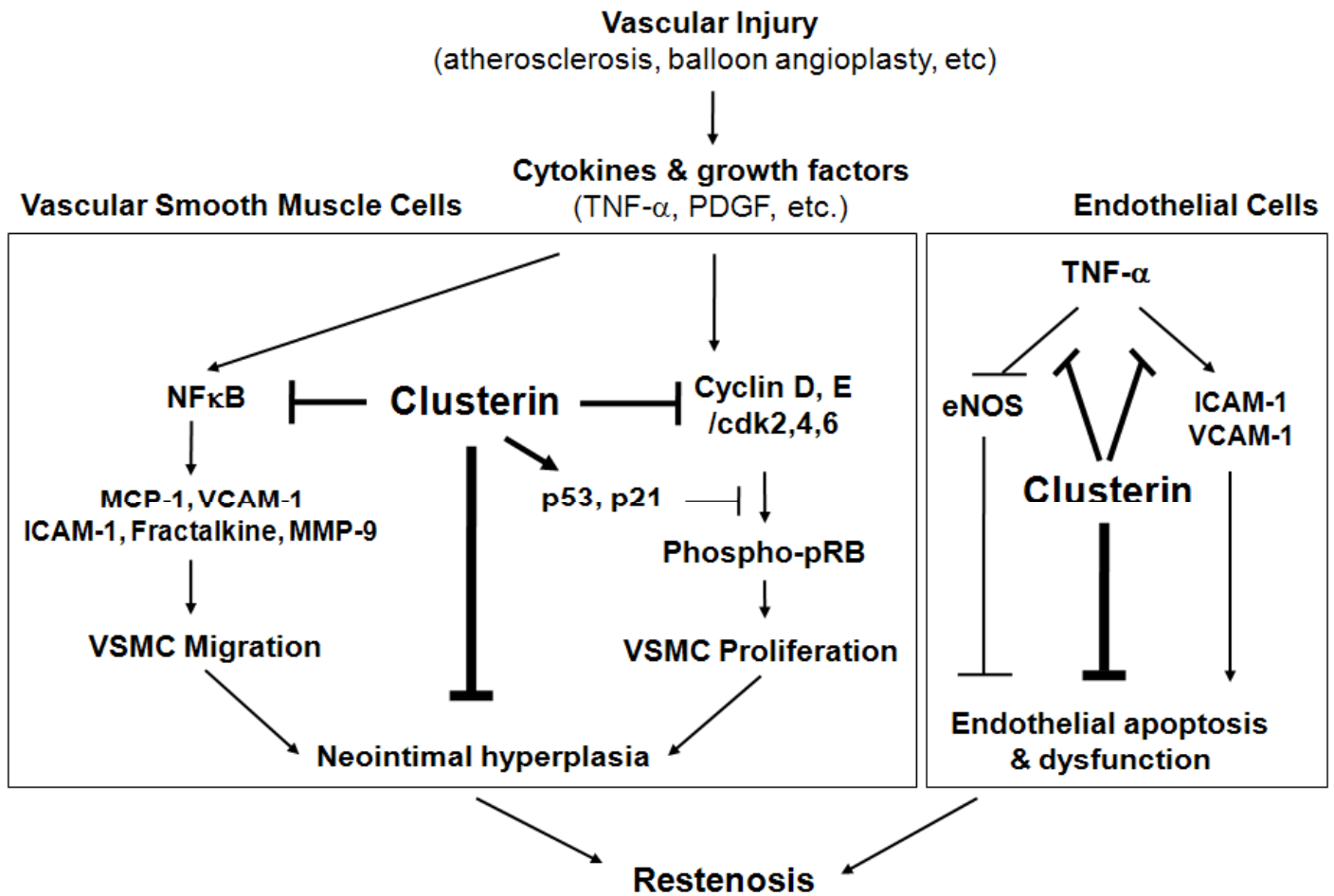

C

p21-pro-Luc

Supplemental Figure IV

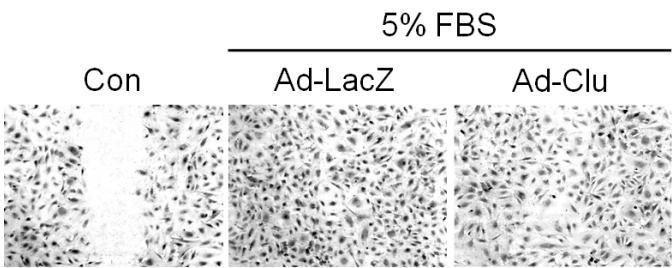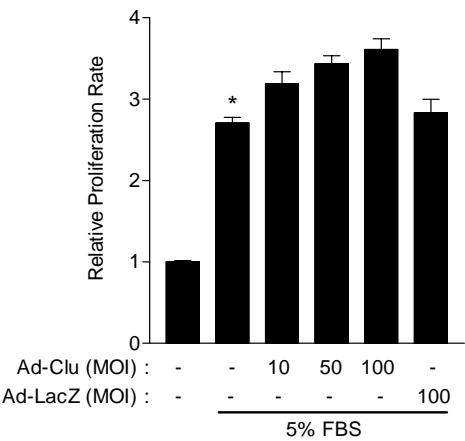**Supplemental Figure V**
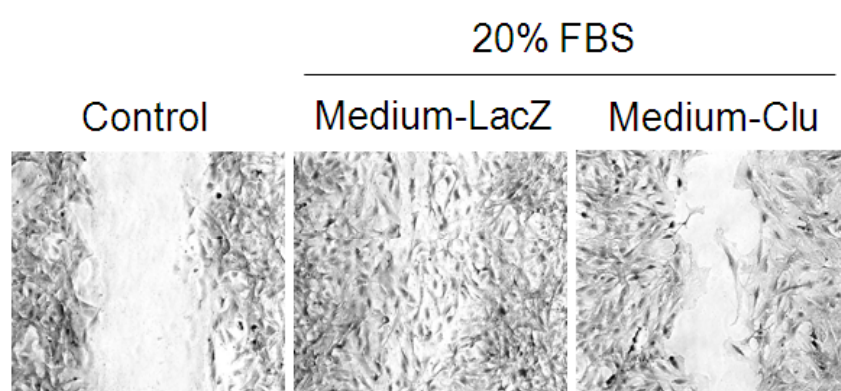
Supplemental Figure VI

Supplemental Figure VII

A

B

Supplemental Figure VIII

Supplemental Figure IX

A

Control
Medium-LacZ
Medium-Clu

Clusterin

β-actin

B

CHX

-     -   Medium-LacZ   Medium-Clu