Matrix Metalloproteinase-9 Is Necessary for the Regulation of Smooth Muscle Cell Replication and Migration After Arterial Injury

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Abstract—Matrix metalloproteinases (MMPs) and, in particular, MMP-9 are important for smooth muscle cell (SMC) migration into the intima. In this study, we sought to determine whether MMP-9 is critical for SMC migration and for the formation of a neointima by using mice in which the gene was deleted (MMP-9−/− mice). A denuding injury to the arteries of wild-type mice promoted the migration of medial SMCs into the neointima at 6 days, and a large neointimal lesion was observed after 28 days. In wild-type arteries, medial SMC replication was ≈8% at day 4, 6% at day 6, and 4% at day 8 and had further decreased to 1% at day 14. Intimal cell replication was 65% at 8 days and had decreased to ≈10% at 14 days after injury. In MMP-9−/− arteries, SMC replication was significantly lower at day 8. In addition, SMC migration and arterial lesion growth were significantly impaired in MMP-9−/− arteries. SMCs, isolated from MMP-9−/− mouse arteries, showed an impairment of migration and replication in vitro. Thus, our present data indicate that MMP-9 is critical for the development of arterial lesions by regulating both SMC migration and proliferation. (Circ Res. 2002;91:845-851.)

Key Words: matrix metalloproteinase-9 ■ mouse arterial injury ■ smooth muscle cell replication ■ migration ■ neointimal formation

Arterial lesion growth is an important process in the development of atherosclerosis, restenosis, and vascular graft stenosis. Multiple events participate in this process, including smooth muscle cell (SMC) replication and SMC migration. The importance of SMC migration is often neglected because in most mammalian arteries, there is no obvious way to recognize the movement of SMCs within the artery. However, the migration of SMCs can be studied in the arteries of small mammals because the intima is composed of just the endothelial monolayer, and in these circumstances, the migration of SMCs to the intima can be evaluated.

The migration of SMCs is thought to be regulated by proteases such as plasminogen/plasmin and matrix metalloproteinases (MMPs). Both classes of enzymes are expressed by SMCs, and their expression is markedly upregulated in injured arteries; these arteries invariably develop a pronounced thickened neointima.1 Plasminogen activators convert the inactive plasminogen to plasmin, which in turn can activate latent MMPs.2 Supporting data that plasmin can regulate SMC migration are given in studies in which the deletion of genes encoding for urokinase plasminogen activator or plasminogen resulted in a smaller intimal lesion size in injured mouse arteries.3,4 There are also convincing data that MMPs are necessary for the migration of SMCs into the intima. The deletion of tissue inhibitor of metalloproteinases (TIMP)-1 in mice leads to the formation of larger intimal lesions in injured arteries, whereas overexpression of TIMP-2 by adenovirus-mediated transfer leads to a temporary reduction in lesion size in injured rat arteries.5,6 Similar data were obtained when a synthetic MMP inhibitor was administered to rats subjected to arterial injury.1,7,8 These arteries showed a significant reduction in the size of the early developing neointima. A concern with both of these approaches is that the inhibitors used are not specific. The synthetic MMP inhibitor used by Bendek et al9 is capable of blocking all MMPs, whereas both TIMP-1 and TIMP-2 can affect the activity of many MMPs. As such, it has not been possible to link a specific MMP with SMC migration and neointimal formation.

Denudation of rat arteries with a balloon catheter leads to an early increase in MMP-9 expression (within 12 hours), accompanied with a later increase in MMP-2 activity.1,7 The fact that MMP-9 expression occurs before any detected SMC migration into the intima suggests that this MMP might be critical for SMC migration. In the present study, we used MMP-9 null (MMP-9−/−) mice to determine what role MMP-9 plays in SMC migration and neointimal thickening. Our results show that not only is SMC migration significantly reduced in MMP-9−/− arteries, but the proliferation of SMC is also impacted. Thus, MMP-9 is a major regulator of neointimal formation.
Materials and Methods

Animals and Surgical Procedure
MMP-9−/− mice and wild-type mice (FVB strain) were kindly provided by Dr Zena Werb (University of California, San Francisco). These mouse colonies were bred in our laboratory, and genotypes were verified by polymerase chain reaction analysis. Adult male mice (25 to 35 g) were anesthetized with an intraperitoneal injection of xylazine and ketamine cocktail (0.14 to 0.2 mg xylazine and 2.1 to 3 mg ketamine). Surgery involved a midline incision on the ventral neck, clearing of the connective tissue around the distal portion of the carotid artery, and placement of a suture loop around the external branch of the carotid above the external/internal bifurcation and another loop distal to external branch. A small incision in the external branch between the 2 suture loops (which are used to control blood flow) was made, and a catheter (made from 6-0 nylon suture) was introduced into the carotid. This catheter was advanced to the aortic arch and then withdrawn with rotation. This procedure was repeated 3 times before removing the catheter. Finally, the external branch was tied off using the sutures in place, and the skin was closed with wound clips. At various times after injury, the mice were killed by intraperitoneal injections of sodium pentobarbital (Abbott Laboratories), and ice-cold lactated Ringer’s solution was infused retrogradely via the left ventricle of the heart. Tissue was infused retrogradely via the left ventricle of the heart.

Zymography
Tissues were ground to a fine powder and collected into lysis buffer containing 50 mmol/L Tris (pH 7.6, Sigma Chemical Co), 1% SDS (Bio-Rad Laboratories), 1 mmol/L phenylmethylsulfonyl fluoride, and 10 μg/mL leupeptin. The samples were centrifuged, and the supernatant was subjected to zymography according to the procedure previously described by Herron et al. Total protein (10 μg) from each extract was loaded onto 8% polyacrylamide gels containing 0.1% type I gelatin (Sigma) for MMP activity. After gel electrophoresis, gels were incubated for 16 to 18 hours at 37°C in a buffer (50 mmol/L Tris [pH 8.0], 2.5 mmol/L CaCl2, and 0.02% sodium azide), rinsed in 10% trichloroacetic acid, and stained in rapid Coomassie stain. The stained gels were visualized by Eagle-Eye Image (Stratagene).

Western Blot Analysis
Arterial tissues were ground to a fine powder and collected into lysis buffer (50 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 50 mmol/L NaCl, 5 mmol/L EDTA, 5 mmol/L EGTA, 2 mmol/L Na3VO4, 0.1% Triton X-100, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 10 μg/mL leupeptin in 10 mmol/L HEPES [pH 7.4]). Total protein (10 μg) was loaded onto a 10% SDS-PAGE gel and transferred onto nitrocellulose membranes (PROTRAN, Schleicher & Schuell). Membranes were blocked with 5% nonfat dry milk in TBS-T (10 mmol/L Tris [pH 8.0], 150 mmol/L NaCl, and 0.1% Tween 20), incubated with primary antibody for 1 hour, washed 3 times with TBS-T, incubated with secondary antibody conjugated to horseradish peroxidase (1:1000 dilution) for 1 hour, washed again, and detected by enhanced chemiluminescence. For detection of cyclin D1 and extracellular signal–regulated kinases (ERKs), rabbit polyclonal antibodies raised against cyclin D1 (1:1000 dilution, Santa Cruz, H-295) and ERKs (1:1000 dilution, New England Biolabs Inc), respectively, were used as primary antibodies.

Histology and Immunohistochemistry
Mice were intraperitoneally injected with bromodeoxyuridine (BrdU, 30 μg/g body wt) at 1, 9, and 17 hours before euthanasia. Carotid arteries were perfusion-fixed with 4% paraformaldehyde in PBS for 3 minutes in situ; this was followed by an additional immersion fixation for 20 minutes. Tissues were transferred to 70% alcohol and then processed for paraffin embedding. Each artery was transversely cut in half, and 2 segments were embedded together in a single paraffin block. Histological sections (5 μm thick in thickness) were cut every 10 sections for staining with hematoxylin and eosin or with Verhoeff–van Gieson. Sections were immunohistochemically stained for smooth muscle α-actin (DAKO), macrophages (MOMA-2), and BrdU (Boehringer-Mannheim). Morphometric analysis of cross-sectional area was performed on arterial sections by using computer-assisted image analysis (NIH Image). Hematoxylin-positive (total) and BrdU-positive (replicating) cells were counted on arterial sections (50 to 100 μm apart), and a total of 8 sections per animal were counted.

En Face SMC Migration Assay
Carotid arteries were perfusion-fixed as described above, and the vessels were opened longitudinally and pinned down onto an agar plate with the luminal surface facing upward. Arteries were stained with hematoxylin for 10 seconds and incubated in Scott’s blue for 2 minutes. Migrated SMCs were identified as the hematoxylin-stained cells on the luminal surface of the endothelium-denuded area. The length of the carotid artery was visualized under a microscope at ×40 magnification (Nikon), and the total number of migrated SMCs was counted.

Mouse Arterial SMC Isolation
Arterial SMCs were isolated from both FVB wild-type and MMP-9−/− mouse aortas by an enzyme dispersion approach. Briefly, the aortas were perfused with lactated Ringer’s solution, and the adventitial tissue was stripped off and then incubated in enzyme mix (2 mg/mL BSA, 1 mg/mL collagenase, 0.375 mg/mL soybean trypsin inhibitor, and 0.125 mg/L elastase type III in Hanks’ balanced salt solution) for 30 minutes at 37°C. After incubation, the adventitial layer was carefully removed using watchmaker forceps. The remaining tissue was minced in a fresh enzyme mix and incubated at 37°C for 2 hours. Digest sample was transferred to a centrifuge tube, spun down, resuspended in 10% FBS + DMEM (high glucose), and plated in a tissue culture flask. Cells were allowed to attach and divide for the next several days.

In Vitro Cell Proliferation and Migration Assays
Arterial SMCs were grown to 70% to 80% confluence, serum-starved for 48 hours, and treated with basic fibroblast growth factor (FGF) for 20 hours and then for 2 hours with the addition of [3H]thymidine (1 μCi/mL) at 37°C. After incubation, [3H]thymidine-containing medium was removed, and cold 10% trichloroacetic acid was added for 10 minutes and then washed with fresh 10% trichloroacetic acid. Then 0.5N NaOH was added, and this DNA-containing NaOH solution was transferred into scintillation vials, and the radioactivity was counted. Arterial SMCs were grown to 70% to 80% confluence and then serum-starved as described above. Cells were trypsinized, washed with PBS, and resuspended in the media (DMEM+0.2% BSA). Approximately 1×104 cells were placed in the upper chamber of a 24-well transwell (Costar) precoated with 0.1% type I collagen, and the medium containing platelet-derived growth factor (PDGF, 20 ng/mL) was added to the lower chamber. After 7 hours of incubation, cells on the lower surface were fixed with methanol and stained with hematoxylin. The transwell membranes were cut out, placed on a glass slide with the bottom surfaces of the membranes facing upward, and mounted with Aquamount. Migrated cells on the lower surface of membranes were counted under a microscope at ×40 magnification.

Results
Time Course of MMP-9 Activity in Mouse Carotid Artery After Injury
MMP-9 activity was undetectable in uninjured mouse carotid arteries; however, a marked increase in MMP-9 gelatinolytic activity was observed within 4 hours after injury, with maximum activity at 24 hours (Figure 1A). MMP-9 activity was still detectable up to 7 days, but no activity was observed at 14 days after injury. In contrast, MMP-2 was constitutively expressed in uninjured carotid arteries, and its activity did not increase until 4 days and remained elevated up to 14 days.
after injury (Figure 1A). Thus, the temporal expression of MMP-2 and MMP-9 in injured mouse arteries is similar to the pattern observed in rat carotid arteries.1 As expected, no MMP-9 activity was detected in MMP-9−/− arteries, and the gelatinolytic activity of MMP-2 was consistently lower in MMP-9−/− arteries (Figure 1B).

Figure 1. A, Gelatinolytic activity of carotid arteries from wild-type (WT) mice at 0, 4, 24, and 48 hours and at 4, 7, and 14 days after injury. NC indicates normal unmanipulated carotid arteries. B, Gelatinolytic activity of carotid arteries in WT mice and MMP-9−/− (KO) mice at 0, 4, and 24 hours after injury. Carotid arteries were collected at various times after injury and were run on a gelatin zymogram.

Response of Mouse Carotid Arteries to Arterial Injury

The technique used for injuring mouse arteries was different from that previously published; so, first, we documented the growth of the neointima in wild-type mouse arteries after injury with a loop of 6-0 nylon. SMCs were present in the intima by 6 days after injury, and 4 weeks later, a marked thickening of the neointima was observed in wild-type arteries (Figure 2). The intimal lesion showed no evidence of macrophages when it was immunostained with macrophage-specific antibody (data not shown). The intimal thickening in mouse arteries was not concentric and was not observed evenly along the common carotid artery. This is different from the intimal thickening pattern observed in rat arteries, where the lesions are symmetrical.1

The neointimal lesion growth was markedly different in MMP-9−/− mice. Neointimal cells were not detectable in most of the mice examined (5 of 8 mice), and a very small thickening was observed in the remaining mice at 4 weeks after injury (Figure 2f). In contrast, the wild-type arteries had large intimal lesions, which markedly reduced the lumen size (Figure 2e). Both the total number of intimal cells and the intimal area were significantly lower in MMP-9−/− mouse carotid arteries than in wild-type arteries, whereas the medial cell number and the medial area were not different (Figures 3A and 3B). These results indicate that early expression of MMP-9 after injury is important for the formation of the neointima.

Figure 2. Time course of arterial lesion development in mice after injury. a, NC artery of WT mouse. b, Carotid artery at 6 days after injury. c, Carotid artery at 8 days after denudation. Note a few layers of neointimal cells. d, Increased intimal thickening at 14 days after injury. e, Substantial neointimal lesion in WT carotid artery at 28 days after denudation. f, Lack of intimal thickening in KO artery at 28 days after injury. Arrowhead marks internal elastic lamina. Each panel is representative of hematoxylin and eosin staining of arterial sections from 5 to 8 animals per time point.

Because MMP-9 can degrade matrix and permit cell migration, we assumed that the difference in the size of the neointima would correlate with a reduced ability of SMCs to migrate into the intima. The SMC migration in MMP-9−/− and wild-type mice was measured at 6 days after injury by counting the number of luminal cells from en face preparations. This time was chosen because SMCs appeared on the luminal surface at 6 days. Approximately 140 SMCs migrated onto the luminal surface of the wild-type mouse carotid arteries, whereas only ~40 cells migrated onto the lumen in MMP-9−/− arteries (Figures 4A and 4B). Thus, MMP-9 is critical for initial SMC migration into the neointima in injured arteries of mice.

Because the difference in neointimal lesions between wild-type and MMP-9−/− mice was so large (see Figure 2), we considered the possibility that MMP-9 might affect SMC replication. In wild-type carotid arteries, the medial SMC replication rate reached ~8% at 4 days and then gradually decreased to ~0.5% at 14 days, and by 4 weeks after injury, no replicating cells were detected (Figure 5A). Intimal SMC replication rate in these arteries was ~65% at 8 day but had
Figure 3. Intimal and medial SMC density (A) and area (B) in WT (solid bar) and KO (hatched bar) carotid arteries at 4 weeks after injury. Both the intimal number of cells and the intimal area are significantly lower in KO mice than in WT mice. An unpaired Student t test was used to test for significant differences between the WT and KO arteries. Data are expressed as mean±SEM. *P<0.05 vs WT.

decreased to 10% at 14 days after injury (Figure 5B). In MMP-9−/− mouse arteries, the early (4-day) SMC replication was similar to that in the wild-type arteries; however, at 8 days after injury, the cell proliferation rate was significantly lower (4.22±1.0% versus 0.98±0.46%, respectively) (Figure 5A). Similarly, the intimal cell replication rate of MMP-9−/− arteries was significantly decreased at 8 days after injury (64.7±6.3% for wild-type arteries versus 41.8±6.9% for MMP-9−/− arteries) (Figure 5B). Furthermore, Western blot analysis showed that cyclin D expression was upregulated at 7 days after injury in wild-type arteries, and this expression was markedly decreased in MMP-9−/− arteries (Figure 5C). These results suggest that MMP-9 gene expression is important for medial and intimal SMC replication, especially at 8 days after injury, and thus contributes to the development of neointimal lesions.

MMP-9−/− and Wild-Type SMC Migration and Proliferation In Vitro
To further examine the role of MMP-9 in SMC migration and proliferation, we isolated medial SMCs from wild-type and MMP-9−/− mouse arteries. The isolated cells were immunostained for α-actin by using an antibody that is specific for SMC α-actin but not for fibroblast α-actin. The result showed that almost 100% of the cells were stained positively for SMC α-actin (data not shown). An in vitro transwell assay for cell migration showed that wild-type SMCs migrated when PDGF was used as a chemoattractant; however, the migration of MMP-9−/− cells was significantly attenuated (Figure 6A). Next, we measured cell replication rates by [3H]thymidine incorporation. An ~3-fold induction in the replication rate was observed with wild-type SMCs when they were treated with FGF, whereas only a 1.3-fold increase in replication rate was detected with MMP-9−/− mouse cells (Figure 6B). In accordance with above data, these in vitro studies clearly indicate that MMP-9 is required in the regulation of arterial SMC migration and proliferation.

Discussion
In a series of experiments examining neointimal lesion formation in injured rat arteries, it became apparent that the migration of SMCs into the intima was a critical event. Early work focused on the role of plasminogen activators and the formation of plasmin as a necessary protease for SMC migration.10,11 Recently, we have become interested in the role of MMPs in neointimal growth. Arteries express MMP-9 within hours after injury and continue to do so for up to 7 days, and this is accompanied with an increase in MMP-2 activation.1 We hypothesized that these MMPs regulate SMC migration, because many metastatic cells express these same proteinases, and we know that SMCs migrate into the intima at times when MMP activity is increased (<7 days). Furthermore, we were able to block SMC migration in injured arteries by the administration of an MMP inhibitor, although the size of the neointimal lesion 14 days after injury was identical to that of injured control arteries.8 This occurred despite the daily administration of the MMP inhibitor.

The findings of the present study are that wild-type mouse arteries express MMP-9 after injury and that there is a significant decrease in SMC migration to the intima in MMP-9−/− arteries. Furthermore, the neointima is signifi-
deletion gives such divergent results. One consideration is that the above-mentioned studies with the MMP inhibitor were carried out in rats and pigs, whereas the present study was in mice. Furthermore, the techniques for injuring arteries in each study were different, although the denuding injuries to rat and mouse arteries appear to elicit similar responses. The pharmacological inhibitor (GM6001) used in these studies has a broad specificity, and one possibility is that this drug interfered with migration in an MMP-independent manner. GM6001 blocks tumor necrosis factor (TNF)-α-converting enzyme, which is needed to release TNF-α from the cell membrane. Therefore, one possibility is that the administration of GM6001 not only blocked the activity of MMPs but also downregulated a TNF-α-induced factor that regulates migration in a negative manner. This event would not occur in mouse arteries with an MMP-9 gene deletion. Finally, there is the possibility that GM6001 does not adequately block MMP-9. The fact that early SMC migration in rats is significantly smaller in MMP-9−/− arteries 28 days after injury. These data confirm our hypothesis that MMPs are critical for regulating SMC migration and that MMP-9 has a dominant role. With respect to this latter issue, we did note that MMP-2 activity was reduced in MMP-9−/− arteries. This fact might suggest that MMP-9 regulates MMP-2 activity directly or indirectly by acting on a certain substrate that can specifically upregulate MMP-2 activity and thus influence SMC migration. Furthermore, because MMP-2 is constitutively expressed in these arteries, changes in MMP-2 activity should occur at the times when MMP-9 is expressed. This is not the case, inasmuch as an increase in MMP-9 was detected by 4 hours, and yet the changes in MMP-2 were not detected until 4 days after injury. We believe that the role of MMP-2 in injured arteries will best be studied by the use of mice with targeted deletion of this gene.

Apart from identifying the critical MMP for SMC migration, these studies also show that a loss of MMP-9 activity can significantly retard neointimal lesion growth. Previous studies in rats and pigs show that MMP inhibition causes only a transient reduction in lesion size and that by 28 days the MMP inhibitor–treated arteries have neointimal lesions equal in size to those of control arteries. These results have been used to imply that MMPs do not play a critical role in neointimal lesion growth. We have no explanation as to why inhibition of MMP-9 by pharmacological agents and by gene
transgenic mice (overexpressing human papilloma virus) led to reduced keratinocyte hyperproliferation and differentiation, whereas Mohan et al. observed an increased epithelial regeneration in the corneas, skin, and tracheas of MMP-9−/− mice. Using doxycycline, a global MMP inhibitor, Bendek et al. showed that intimal but not medial SMC replication at 7 days was inhibited in injured rat arteries. This latter result matches well with our findings of a decrease in SMC replication that was detected in MMP-9−/− mouse arteries at late (at day 8) but not early times after injury. However, in our experiment, both medial and intimal SMCs were inhibited.

How MMP-9 regulates entry into the cell cycle is unclear. One explanation could be that MMP-9 releases matrix-bound cytokines and growth factors. These might include FGF, vascular endothelial growth factor, transforming growth factor-β, and TNF-α. Of these, basic FGF (FGF2) is a likely candidate. Indeed Sasaki et al. reported that NO via activation of MMP-9 and MMP-2 was responsible for the release of FGF2. FGF2 is liberated after injury to arteries and has a well-documented affinity for molecules of the extracellular matrix of arteries. Our data in rat injured arteries show that FGF2 is a potent mitogen for SMCs and that its chronic administration induces large intimal lesions. Thus, the release of sequestered FGF by MMP-9 might explain the marked difference in SMC replication at 8 days after injury. In wild-type arteries, MMP-9 would be able to free matrix-bound FGF2 and thus stimulate further cell replication, but this would not occur in MMP-9−/− arteries. By day 14, however, SMC replication in the wild-type arteries is equal to that in the MMP-9−/− arteries, presumably because at this time MMP-9 activity is downregulated. Alternatively, this could be due to depletion of the pool of extracellular FGF2. We are now examining the possibility that MMP-9 can release matrix-bound FGF2.

Another possible mechanism of how MMP-9 may regulate SMC replication is by disrupting cell-to-cell interactions in injured arteries. A recent study by Ho et al. showed that overexpression of TIMP-1 or direct inhibition of MMPs upregulates cadherin expression as well as cell-to-cell adhesion in fibroblasts. This was associated with a reduction in the ability of cells to show signs of transformation. Cadherin is a transmembrane glycoprotein that associates with α, β, and γ catenins. Loss of cadherin from cell membrane releases β-catenin, which translocates to the nucleus and, in association with the transcription factor, LeF1/Tcf, regulates several genes, including cyclin D1. Therefore, it is possible that MMP-9 may regulate SMC growth by modulating β-catenin and cadherin association. We have observed that LeF1 and β-catenin are highly expressed at times of increased cell proliferation in injured rat arteries. Furthermore, we have preliminary data showing an increased association of β-catenin with E-cadherin in SMCs isolated from MMP-9−/− mice (data not shown).

There are reports that other MMPs may regulate intimal lesion growth in mouse arteries. For example, inactivation of the MMP-11 gene was shown to increase neointimal formation and elastin degradation after perivascular electrical arterial injury. However, this injury caused little SMC replication but was accompanied with a significant inflammatory response. As such, it is likely that MMP-11 was derived from invading macrophages and was unrelated to SMC migration and replication. In addition, inactivation of the MMP-3 gene was shown to reduce aneurysm formation, but it had no effect on the progression of atherosclerotic lesions in apoE null mice. In the present study, neither MMP-3 nor MMP-11 was detected, presumably because of the absence of inflammatory cells; therefore, it is unlikely that they play any role in the formation of neointimal lesions after a denuding injury.

The mice used in the present study were of FVB background strain. This strain forms a large intimal lesion after arterial injury and thus allows us to document the effect of inactivation of the MMP-9 gene in injured arteries. Harmon et al. reported that compared other mouse strains, FVB mice possess much higher proliferative response to flow-reduced ligation model; in fact, Harmon et al observed large differences in intimal lesion formation between 11 different mouse strains. Recent work by Kuhel et al. has documented that C57Bl/6 mice are resistant to neointimal hyperplasia, whereas FVB/N mice are susceptible to neointimal hyperplasia after arterial injury. In addition, we have preliminary data showing that neointimal formation and SMC replication are greater in FVB mice than in C57Bl/6 mice. Thus, genetic background appears to plays an important role in the formation of intimal lesions, and linkage analysis studies would help to understand the influence of genetic makeup on the arterial tissue response to injury.

In conclusion, we have presented evidence that MMP-9 is critical for the development of neointimal lesions in injured arteries and that MMP-9 contributes to arterial lesion growth by regulating both SMC migration and replication. Thus, MMPs appear to possess multiple functional abilities that are important for vascular tissue remodeling.

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References
Cho and Reidy

MMP-9 Regulates Arterial Lesion Growth

851


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