Article Type: Original Article

Subject category: Health Services and Outcomes Research

A membrane-tethered metalloproteinase expressed by vascular smooth muscle cells limits the progression of proliferative atherosclerotic lesions

Running title: *Barnes et al.; VSMC MT1-MMP limits atherogenesis and aneurysm*

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Journal Subject Terms: Atherosclerosis, Aneurysm, Vascular Biology, Genetically Altered and Transgenic Models, Smooth Muscle Proliferation and Differentiation

Abstract

Background: The matrix metalloproteinase (MMP) family plays diverse and critical roles in directing vascular wall remodeling in atherosclerosis. Unlike secreted-type MMPs, a member of the membrane-type MMP family, MT1-MMP (MMP14), mediates pericellular extracellular matrix (ECM) degradation that is indispensable for maintaining physiological ECM homeostasis. However, due to the premature mortality exhibited by MT1-MMP-null mice, the potential role of the proteinase in atherogenesis remains elusive. We therefore sought to determine the effects of both MT1-MMP heterozygosity and tissue-specific gene targeting on atherogenesis in APOE-null mice.

Methods and Results: MT1-MMP heterozygosity in the APOE-null background (*Mmp14*$^{+/-}$ *Apoe*$^{-/-}$) significantly promoted atherogenesis relative to *Mmp14*$^{+/+}$*Apoe*$^{-/-}$ mice. Further, the tissue-specific deletion of MT1-MMP from vascular smooth muscle cells (VSMCs) in SM22α-Cre(+)*Mmp14*$^{F/F}$ *Apoe*$^{-/-}$ (VSMC-KO) mice likewise increased the severity of atherosclerotic lesions. While VSMC-KO mice also developed progressive atherosclerotic aneurysms in their iliac arteries, macrophage- and adipose-specific MT1-MMP knockout mice did not display this sensitized phenotype. In VSMC-KO mice, atherosclerotic lesions were populated by hyper-proliferating VSMCs (SMA- and Ki67- double positive cells) that were characterized by a pro-inflammatory gene expression profile. Finally, MT1-MMP-null VSMCs cultured in a 3-D spheroid model system designed to mimic *in vivo*-like cell-cell and cell-ECM interactions, likewise displayed a markedly increased proliferative potential.

Conclusions: MT1-MMP expressed by VSMCs plays a key role in limiting the progression of atherosclerosis in APOE-null mice by regulating proliferative responses and inhibiting the deterioration of VSMC function in atherogenic vascular walls.

Key words: matrix metalloproteinases; inflammation; atherosclerosis; aneurysm; muscle, smooth

Clinical Perspective

What is new?

- The molecular mechanisms underlying proliferative atherosclerosis have not been fully defined.
- This animal study suggests that a pericellular collagenase, called membrane-type 1 matrix metalloproteinase (MT1-MMP), expressed by vascular smooth muscle cells (VSMCs) plays a critical role in limiting the progression of proliferative atherosclerotic lesions.
- The loss of VSMC MT1-MMP leads to advanced proliferative atherosclerosis and atherosclerotic iliac artery aneurysm formation.

What are the clinical implications?

- The functional impairment of VSMCs in regulating vascular wall extracellular matrix remodeling may contribute to the pathogenesis of proliferative atherosclerosis and atherosclerotic iliac artery aneurysm formation.

Vascular smooth muscle cells (VSMCs) constitute the major cellular component of the tunica media where they play key roles in regulating vascular tone and blood flow [1]. Under physiological conditions, the number of vascular smooth muscle cells within the arterial wall is tightly controlled, as is arterial wall thickness [1, 2]. In marked contrast, during the progression of atherosclerosis, VSMCs proliferate and transition from a contractile to a synthetic phenotype, thereby depositing excess extracellular matrix (ECM) molecules that lead to arterial wall thickening and stiffening, i.e., arteriosclerosis [3, 4]. However, the molecular mechanisms that underlie VSMC proliferation and arteriosclerosis during the pathological process of atherosclerosis remain largely undefined.

ECM remodeling is mediated by members of the matrix metalloproteinase (MMP) gene family, a group of structurally-related proteolytic enzymes that are broadly characterized as either secreted or membrane-tethered [5]. Consistent with their potential roles in vascular wall pathology, recent studies have characterized the roles of secreted MMPs, particularly MMP-2, MMP-8 and MMP-13, in promoting atherogenesis or plaque rupture [6-8]. With regard to the membrane-tethered MMPs, MT1-MMP uniquely serves as a pericellular collagenase that

cleaves native, triple-helical collagens associated with both the basement membrane and interstitial matrix [9]. Indeed, in contrast to almost all other MMP family members wherein gene targeting exerts only minor effects on mouse development, MT1-MMP knockout animals display severe dwarfism, lipodystrophy and premature lethality, underscoring the key roles played by MT1-MMP in maintaining tissue homeostasis [10-12]. Due to the premature lethality of MT1-MMP knockout mice, however, attempts to characterize the role of MT1-MMP in atherogenesis have been limited. To date, Schneider et al. used a bone marrow transplant system to transfer MT1-MMP-null bone marrow cells into atherogenic *Ldlr*$^{-/-}$ mice to assess the role of the proteinase when expressed by myeloid cells in atherogenesis [13]. The reconstitution of atherogenic mice with MT1-MMP-null bone marrow cells did not alter the size of the atheroma or the number of infiltrating macrophages, but did result in an increased collagen content within the atheromatous lesions [13]. Hence, while myeloid cell-derived MT1-MMP appears to control collagen turnover, the role of MT1-MMP in regulating atherogenic responses in non-myeloid cell populations remains unknown.

Despite the paucity of information regarding the role of MT1-MMP in atherogenesis, MT1-MMP heterozygous mice, which do not display any of the severe developmental phenotypes observed in the MT1-MMP-null mice, have been reported to display a protected status from both high fat diet (HFD)-induced obesity [14] and neointima formation secondary to carotid injury [15]. Given the attenuated responses of MT1-MMP heterozygous animals to pathologic stresses, we initially hypothesized that these mice might likewise be protected from hypercholesterolemic atherogenesis. Contrary to our expectations, we now report that atherogenesis is significantly enhanced in heterozygous *Mmp14*$^{+/-}$*Apoe*$^{-/-}$ mice. To determine the cellular mechanisms by which MT1-MMP limits the progression of atherogenesis, we have extended our analyses to include newly characterized conditional knockout mice to selectively target MT1-MMP expression in adipose tissue, macrophages, or VSMCs in APOE-null mice. Unexpectedly, we find that MT1-MMP expressed by VSMCs, and not by either adipocytes or macrophages, exerts a profound protective effect against the progression of proliferative atherosclerotic lesions in *Apoe*$^{-/-}$ mice. Together, these results constitute the first example of MT1-MMP serving as an anti-atherogenic enzyme by directly regulating VSMC function and proliferation in the *in vivo* setting.

Material and methods

Animals: *Apoe*$^{-/-}$ mice [16] were purchased from the Jackson laboratory (JAX, stock number 002052). MT1-MMP heterozygous *Mmp14*$^{+/-}$ mice were maintained on a C57BL6/J background with more than 5 generations of backcrossing [14, 17]. *Mmp14*$^{+/-}$ mice were crossed to *Apoe*$^{-/-}$ mice to generate *Mmp14*$^{+/-}$*Apoe*$^{+/-}$ breeders. *Mmp14*$^{+/-}$*Apoe*$^{+/-}$ mice were used for breeding with *Apoe*$^{-/-}$ mice to generate *Mmp14*$^{+/-}$*Apoe*$^{-/-}$ mice and their littermate *Mmp14*$^{+/+}$*Apoe*$^{-/-}$ mice for this study. *Mmp14*$^{F/F}$ mice were generated as described previously [18]. These mice were crossed to *SM22α-Cre* [JAX, Tg(Tagln-cre)1Her/J, stock number 004746] [19], *Csf1r-Cre* (JAX 019098) or *Fabp4-Cre-ERT2* transgenic mice (gift from Pierre Chambon, IGBMC) [20], then further crossed to *Apoe*$^{-/-}$ mice.

Atherosclerosis study

The atherogenic Western diet, comprised of 17% (kcal/kcal) protein, 43% carbohydrate, and 41% fat with 1.5 g/kg cholesterol, was purchased from Research Diets (D12079B). Male and female mice were fed a Western diet for 12 weeks beginning at 8 weeks of age. All procedures were approved by the University of Michigan Committee on the Use and Care of Animals, conforming to the International Guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care.

Morphometric analysis of atherosclerosis

After euthanasia by CO_2 asphyxiation, blood was collected through portal veins. Animals were perfused with phosphate buffered saline (PBS) and then 10% formalin in PBS through their left ventricles at a rate of 1 ml/min as described previously [21]. Arterial trees were carefully dissected to include the brachiocephalic, left common carotid and subclavian arteries, as well as the descending thoracic and abdominal aortas with the bilateral iliac arteries. Adipose tissues attached to the arteries were carefully removed under a dissecting microscope. After Oil-Red-O staining and repeated washing in ethanol and water, aortic trees were pinned against a black background, and the aortic trees were digitally photographed under a dissecting microscope [22]. Oil-Red-O positive areas were quantified as the % plaque area per total arterial tree or abdominal aorta.

Collagen staining and histological analysis

Sections were stained with hematoxylin and eosin (HE) and Sirius Red [23]. The atheroma area and vascular wall area were quantified in cross-sections using ImageJ software (N.I.H.), and the average plaque and vascular wall areas were determined. Immunostaining was performed using the ABC system (Vector). The primary antibodies used were rabbit polyclonal anti-alpha smooth muscle actin antibody (Abcam, ab5694), anti-F4/80 antibody (Santa Cruz Biotechnologies, sc-26643), and anti-Ki67 antibody (Abcam, ab15580).

Vascular smooth muscle cells

Mouse aortic smooth muscle cells were isolated with collagenase digestion as described by others [24]. Isolated primary mouse VSMCs were cultured in high-glucose DMEM (ThermoFisher) with 10% fetal bovine serum (FBS, Hyclone) and passed twice before experiments. Mouse primary vascular smooth muscle cells immortalized with the SV40 large T antigen were purchased from ATCC (MOVAS, CRL-2797) [25].

3-D spheroid culture

Cells (2×10^4) were cultured as hanging droplets in a 384-well Perfecta3D hanging droplet plate (3D Biomatrix) for 48 hours before assays [26]. Cells were cultured in high-glucose DMEM with 10% FBS medium supplemented with 0.24% Methocel A4M (Sigma-Aldrich).

Whole genome expression analysis

The total RNA was extracted from cultured primary VSMCs. Two independent pairs of samples were used for the DNA microarray experiments. Labeled cRNA was hybridized to Affymetrix Mouse Gene ST 2.1 strips at the University of Michigan DNA Sequencing Core. Expression values were calculated using a robust multi-array average (RMA). Probe sets that showed more than two-fold difference between the groups with a minimal expression value > 2^4 in at least one of the samples were chosen for further statistical analyses. Data analysis was performed with R version 3.1.1, and a heat map was created using the “gplots” package (R package version 2.17.0. http://CRAN.R-project.org/package=gplots). Significant biological pathways represented by the differentially expressed genes were determined using PANTHER (http://pantherdb.org/).

Statistical data analyses

Values were expressed as the means ± SEM. The distribution of weight, fat mass, cholesterol profile, fasting insulin and glucose levels between two groups were analyzed using two-tailed, unpaired Student t-test. To assess the differences in atherogenesis between the multiple groups, two-way ANOVA was used, followed by a post-hoc pairwise comparison using Turkey's procedure. *P* values below 0.05 were considered to be significant.

Results

The effect of the MT1-MMP gene dose on fat mass and dyslipidemia in APOE-null mice

We previously demonstrated that MT1-MMP heterozygosity renders mice resistant to HFD-induced obesity [14]. Based on this finding, we hypothesized that the allelic reduction of the MT1-MMP gene would protect mice from hypercholesterolemic atherosclerosis, a disease process that is often associated with obesity in humans [27]. As such, we crossbred MT1-MMP heterozygous (*Mmp14*$^{+/-}$) mice with APOE-null mice to generate *Mmp14*$^{+/-}$*Apoe*$^{-/-}$ mice and examined the effects of MT1-MMP heterozygosity on weight, fat mass, blood glucose and insulin levels as well as cholesterol profiles. As expected, heterozygous *Mmp14*$^{+/-}$*Apoe*$^{-/-}$ (HT) male mice on a Western diet were leaner than littermate wild-type *Mmp14*$^{+/+}$*Apoe*$^{-/-}$ (WT) mice (WT 33.0±1.0 g, HT 29.0±0.9 g, n = 9 each, *P* = 0.009, Fig. 1A). When fat mass was assessed at the end of 12-week Western diet, the total and epididymal fat masses were significantly smaller in the *Mmp14*$^{+/-}$*Apoe*$^{-/-}$ than *Mmp14*$^{+/+}$*Apoe*$^{-/-}$ male mice (% total fat mass per weight, WT 5.1±0.3%, HT 3.6±0.6%, n = 9 each, *P* = 0.03; % epididymal fat per weight, WT 3.4±0.2%, HT 2.2±0.3%, n = 9 each, P=0.007, Fig. 1B). Fasting blood glucose levels were similar between the groups (WT 162±43 vs. HT 161±27 mg/dL, n = 8 and n = 9, respectively, *P* = 0.5); however, the fasting insulin concentration was substantially lower in the MT1-MMP heterozygous mice (WT 11.8±4.3 vs. HT 3.8±0.6 mU/L, n = 8 and n = 9, respectively, *P* = 0.04), suggesting an increased insulin sensitivity of *Mmp14*$^{+/-}$*Apoe*$^{-/-}$ mice that occurs in parallel with their leaner phenotype. Of note, MT1-MMP heterozygosity did not change the blood cholesterol levels in APOE-null mice fed a Western diet (total cholesterol, WT 1021±59 vs. HT 1012±52 mg/dL, n = 8 and n = 9, respectively, *P* = 0.9; direct LDL, WT 359±27 vs. HT 349±23 mg/dL, *P* = 0.8, Fig. 1C). Consistent with their leaner phenotype and relative insulin sensitivity, the blood triglyceride content tended to be lower in HT mice, but this difference did not reach statistical significance (WT 142±10 mg/dL, HT 118±11 mg/dL, n = 8 and n = 9, respectively, *P* = 0.1).

Whole-body MT1-MMP heterozygosity accelerates atherogenesis

MT1-MMP WT and HT mice were fed a Western diet for 12 weeks beginning at 8 weeks of age. Contrary to our prediction, atherogenesis was more advanced in *Mmp14*$^{+/-}$*Apoe*$^{-/-}$ mice compared to *Mmp14*$^{+/+}$*Apoe*$^{-/-}$ littermate controls (Fig. 1D). Particularly, advanced atherogenesis was notable in the abdominal aorta and iliac/femoral arteries of *Mmp14*$^{+/-}$*Apoe*$^{-/-}$ mice (Fig. 1D arrows). The % total plaque area was increased by 21% in male mice (*Mmp14*$^{+/+}$*Apoe*$^{-/-}$ 14.9±0.9% vs. *Mmp14*$^{+/-}$*Apoe*$^{-/-}$ 18.1±0.8%, n = 7 each, $P = 0.04$, Fig. 1E, left), while the abdominal % plaque area was increased by 200% (2.3±0.5% vs. 6.9±0.5%, n = 7 each, $P < 0.0001$, Fig. 1E, right). In female mice, the total plaque area in *Mmp14*$^{+/-}$*Apoe*$^{-/-}$ animals was increased by 56% (15.1±1.3% vs. 23.6±1.6%, n = 4 and n = 10, respectively, $P = 0.002$, Fig. 1F, left), with the abdominal plaque area increasing by 171% relative to the *Mmp14*$^{+/+}$*Apoe*$^{-/-}$ female controls (3.5±0.2% vs. 9.5±1.8%, n =4 and n= 10, respectively, $P = 0.002$, Fig. 1F, right).

We next examined left carotid artery sections to assess the structure of the atherosclerotic plaque and the associated level of vascular wall remodeling. Interestingly, the average plaque area of the *Mmp14*$^{+/-}$*Apoe*$^{-/-}$ mice was three times as large as that of the *Mmp14*$^{+/+}$*Apoe*$^{-/-}$ mice, but this was not statistically significant due to the variable severity of atherosclerosis in the heterozygous group (WT 59±7 x10^3 μm^2 vs. HT 189±84 x10^3 μm^2, n = 6 each, $P = 0.15$, Fig. 2A). Concurrent with a trend of increased atherosclerosis, we observed a 1.7-fold increase in the vascular wall area, underscoring the presence of outward vascular wall remodeling in MT1-MMP heterozygous APOE-null mice (133±16 x10^3 μm^2 vs. 234±19 x10^3 μm^2, n = 6 each, $P = 0.004$). Furthermore, both the atherosclerotic plaques and the vascular walls of MT1-MMP HT mice displayed an increased collagen fiber content relative to WT mice as assessed by Sirius Red staining (Fig. 2B, Sirius Red-positive area, WT 26.6±3.3 vs. HT 44.0±4.7%, n = 6 each, $P = 0.009$), suggesting that MT1-MMP heterozygosity promoted vascular wall arteriosclerosis along with plaque formation in the APOE-null background. MT1-MMP HT mice also displayed an apparent loss of a contractile phenotype (i.e., loss of SMA staining) coupled with disrupted elastic lamina, which in turn, elicited more complex and advanced atherosclerotic lesions (Fig. 2C, D).

MT1-MMP gene targeting in VSMCs accelerates atherosclerosis progression

While MT1-MMP heterozygosity was found to promote atherogenesis, the cellular mechanisms underlying the aggravated atherogenesis were unclear. In obesity, perivascular adipose tissues play a key role in atherogenesis [28]. On the other hand, dysfunctional VSMCs might also be responsible for inducing inflammatory atherogenesis [4]. As MT1-MMP is highly expressed in both adipocytes [9, 10] and VSMCs [15], we sought to use tissue-specific knockout mice in an effort to determine the cell type responsible for the MT1-MMP-dependent regulation of atherogenesis. When male mice in each group were fed a Western diet, we observed markedly augmented atherogenesis and aneurysm formation only with VSMC-specific MT1-MMP gene targeting, i.e., *SM22α-Cre(+)Mmp14$^{F/F}$Apoe$^{-/-}$* mice, but not following adipocyte MT1-MMP gene targeting, i.e., *aP2-Cre-ERT2(+)Mmp14$^{F/F}$Apoe$^{-/-}$* mice (Fig. 3A). Atherogenesis in *SM22α-Cre(+)Mmp14$^{F/F}$Apoe$^{-/-}$* mice frequently extended to femoral arteries with significant outward remodeling having been noted, in tandem with aneurysm formation in the iliac arteries (Fig. 3A, B). Importantly, total plaque area was increased by 190% in *SM22α-Cre(+)Mmp14$^{F/F}$Apoe$^{-/-}$* male mice compared with *Cre(-)Mmp14$^{F/F}$Apoe$^{-/-}$* male mice (8.0±1.4 vs. 23.5±1.4%, n = 8 and n = 13, respectively, P<0.0001) and 210% in female mice (4.9±0.9 vs.15.5±3.5%, n = 5 and n = 7, respectively, P=0.03). By contrast, significant differences were not observed between Cre-negative controls and the adipose-specific MT1-MMP deletion model (Fig. 3C and Figure S1), suggesting that the VSMCs are the primary cell type that mediates MT1-MMP-dependent modulation of atherogenesis progression.

The loss of VSMC MT1-MMP leads to proliferative atherosclerotic lesions and aneurysm formation

After 12-week Western diet feeding, SM22α-Cre(+)*Mmp14$^{F/F}$Apoe$^{-/-}$* mice developed extensive atherosclerosis in their common iliac arteries followed by aneurysm formation (Fig. 4A, B). No aneurysm formation was observed in the *Cre(-)Mmp14$^{F/F}$Apoe$^{-/-}$* mice (8 male and 5 female mice), whereas 11 out of 12 male and 6 out of 7 female *SM22α-Cre(+)Mmp14$^{F/F}$Apoe$^{-/-}$* mice developed strikingly enlarged aneurysms that were readily observable upon dissection (Fig. 3B). These dysmorphic lesions displayed significant vascular wall thickening as well as atheroma formation (Fig. 4A). Masson Trichrome and VVG staining demonstrated the disruption and loss of elastic laminae (Fig. 4A). SMA staining showed an increased number of SMA+ cells in vascular walls as well as within atheroma where Ki67 staining confirmed that large numbers of SMA+ cells existed in a proliferative state (Fig. 4A, D). Infiltration of F4/80-positive cells was observed in the atheroma and the vascular walls of affected vessels (Fig. 4A, F4/80 staining),

but to a lesser extent compared with VSMCs. Image quantification of the lesions confirmed an increased atheroma area (Fig. 4B) and outward remodeling (Fig. 4C), which were coupled with an increased number of proliferating VSMCs (Fig. 4D). As macrophages are potentially targeted by SM22α promoter-driven Cre expression [29], we specifically targeted these cells using *Csf1r-Cre(+)Mmp14$^{F/F}$Apoe$^{-/-}$* mice and examined atherosclerosis and aneurysm formation. Neither littermate control *Csf1r-Cre(–)Mmp14$^{F/F}$Apoe$^{-/-}$* mice nor *Csf1r-Cre(+)Mmp14$^{F/F}$Apoe$^{-/-}$* mice developed atherosclerotic iliac aneurysm after 12 weeks of Western diet (n=7 for each group tested), and no significant differences in the sizes of atherosclerosis lesions were observed (n=4, each).

Proinflammatory and metabolically dysfunctional MT1-MMP-null VSMCs

To assess the role of MT1-MMP in VSMC function, we isolated primary VSMCs from descending aortas. As expected, VSMCs isolated from *SM22-Cre(+)Mmp14$^{F/F}$Apoe$^{-/-}$* mice demonstrated specific Cre expression along with the suppression MT1-MMP gene expression (Figure S2). *In vitro*, MT1-MMP-null VSMCs displayed a flattened, spread shape with higher stress fiber formation (Fig. 5A), while the expression of other MMPs, e.g., MMP-2, -8, -9, -13, and MT2-MMP (MMP15), were not significantly different between control and MT1-MMP-deleted cells (Figure S2). Acta2 gene, which encodes α-SMA, was expressed equally in primary VSMCs isolated from *SM22-Cre(-)Mmp14$^{F/F}$Apoe$^{-/-}$* and *SM22-Cre(+)Mmp14$^{F/F}$Apoe$^{-/-}$* mice (Figure S2). To gain further insight into the effects of MT1-MMP gene targeting in VSMCs, whole genome transcriptome analysis was performed. Using a minimum two-fold difference as a cutoff, 414 genes were found to be differentially expressed between the two groups (the top 70 genes are shown in Fig. 5B and all differentially expressed genes are listed in Table S1). Interestingly, the genes upregulated in *SM22α-Cre(+)Mmp14$^{F/F}$Apoe$^{-/-}$* VSMCs (>2.3-fold change) were aggregated in the pathways of inflammation and cell killing (Fig. 5C), suggesting the acquisition of a proinflammatory phenotype in MT1-MMP-null VSMCs.

MT1-MMP gene targeting promotes VSMC proliferation in 3-D cell-cell and cell-ECM contexts

To gain insights into the mechanism by which MT1-MMP-null VSMCs engage in the formation of proliferative atherosclerotic lesions, we developed an *in vivo*-like 3-D spheroid culture system

wherein *in vivo*-like cell-cell and cell-ECM interactions could be recapitulated. Using Ki67 as an index of proliferative responses, MT1-MMP-null primary VSMCs displayed markedly higher activity relative to wild-type cells (Fig. 6A, B). To determine whether the observed differences in cell proliferation were a direct consequence of MT1-MMP gene targeting (i.e., as opposed to a secondary response of VSMC recovered from the advanced atherogenic lesions found in VSMC-KO mice), we used immortalized mouse primary VSMCs (MOVAS) to further define the role of MT1-MMP in regulating VSMC proliferation. In 3-D spheroid culture, similar to the findings obtained with freshly isolated VSMCs, siRNA-mediated MT1-MMP gene silencing in MOVAS induced a marked increase in the number of Ki67-positive VSMCs (Fig. 6C, D). Of note, under 2-D culture conditions, MT1-MMP silencing exerted minimal effects on MOVAS proliferation (Fig. 6E and F). Taken together, these results support a model wherein MT1-MMP plays a required role in regulating VSMC proliferative activity, but only under 3-D conditions that more closely recapitulate the *in vivo* environment.

Discussion

MMP family members play a key role in ECM turnover in a wide variety of developmental and disease processes [30]. Among the collagen-degrading MMPs, secreted (MMP-3, -8, -13) and membrane-type MMPs (MT1- and MT2-MMP) display distinct temporospatial differences in their patterns of expression and activity [8]. Unlike other MMPs, MT1-MMP (MMP14) is the only family member whose activity is indispensable for postnatal development [9, 10]. Due to the premature morbidity and mortality displayed by MT1-MMP-null mice, the role of the proteinase in cardiovascular disease has remained elusive. To date, the only MT1-MMP-expressing cellular compartment tested in a mouse atherogenesis model was bone marrow-derived myeloid cells; however, no substantial impact on atheroma size were observed following bone marrow reconstitution with *Mmp14* $^{-/-}$ cells [13]. In our study, we have identified the critical role played by VSMC-derived MT1-MMP in regulating the progression of atherosclerotic lesions and the associated formation of vascular aneurysms.

Previously, we demonstrated that MT1-MMP heterozygosity protects C57BL/6 mice from diet-induced adipose tissue expansion [14]. Similarly, in this study, heterozygous *Mmp14*$^{+/-}$ mice displayed a leaner phenotype relative to *Mmp14*$^{+/+}$ mice in an APOE-null background. Consistent with their leaner phenotype, MT1-MMP heterozygous mice displayed higher insulin sensitivity than WT mice. Given the metabolically improved status of *Mmp14*$^{+/-}$ mice, we initially

hypothesized that MT1-MMP heterozygosity would protect mice from hypercholesterolemic atherosclerosis, which is often associated with increased adiposity. Contrary to our expectations, MT1-MMP heterozygous APOE-null mice developed more extensive atherosclerotic lesions than MT1-MMP-sufficient APOE-null mice, suggesting a potentially beneficial role for MT1-MMP in limiting disease progression. The results also suggest that the inverse relationship observed between fat mass and atherosclerosis, i.e., the so-called "obesity paradox" in humans, may reflect the independent biological effects exerted by a cohort of modifier genes, including MT1-MMP, on adipose tissue and vascular function.

To define the cellular mechanisms by which MT1-MMP exerts its anti-atherogenic effects, we embarked on a series of studies aimed at tissue-specific MT1-MMP gene targeting in APOE-null mice. In the early stages of our efforts, we focused on two cell types – adipocytes and VSMCs. We initially hypothesized that the loss of adipocyte MT1-MMP would modify atherogenesis via the potentially causal links that exist between adipose tissue and the vascular wall [31]. In APOE-null mice, however, we were unable to observe a significant impact on atherogenesis following gene targeting of adipocyte-derived MT1-MMP. By contrast, the SM22α-Cre-mediated loss of VSMC MT1-MMP strikingly aggravated atherosclerosis progression in APOE-null mice. Whole-genome transcriptome analyses indicated that a series of proinflammatory genes were upregulated in MT1-MMP-null VSMCs, a finding consistent with the pro-atherogenic phenotype of *SM22α-Cre(+)Mmp14$^{F/F}$Apoe$^{-/-}$* mice. Furthermore, our *in vitro* studies demonstrated that MT1-MMP targeting in either primary VSMCs or immortalized VSMCs promotes cell proliferation under 3-D spheroid culture conditions [26]. As such, 3-D spheroid culture appears to reflect a set of conditions that better reflect our *in vivo* findings and as such, can be used to more faithfully address cellular behavior in tissue-like contexts by recreating cell-cell and cell-ECM interactions *ex vivo*. Indeed, previous work has demonstrated that VSMC proliferative activity can be regulated by cell-cell adhesion [32-34] and cell-ECM interactions [34-36]. Of note, the enhanced proliferative responses displayed by MT1-MMP-null VSMCs were not observed under conventional 2-D culture conditions where the cell-cell and cell-ECM interactions that are encountered *in vivo* are replaced by cell culture atop a non-physiologic, planar and rigid substratum. Finally, it is interesting to note that the proliferative effects of MT1-MMP gene silencing were not observed in other cell types, e.g., 3T3-L1 preadipocytes (data not shown), reinforcing the unique role played by MT1-MMP in VSMC biology.

In considering the mechanisms by which MT1-MMP might control proliferative activity, efforts are complicated by the proteinase's broad substrate repertoire, ranging from type I collagen [37] to CD44 [38] and cadherins [39]. As such, MT1-MMP can potentially regulate VSMC proliferation by degrading any number of membrane-associated protein targets as well as pericellular ECM molecules, thereby modifying both cell-cell adhesion and cell-ECM interaction. In atherosclerosis, the expression of adhesion molecules and ECM proteins are highly upregulated [40], as such, a decrease in MT1-MMP activity would be predicted to trigger the excess accumulation of ECM macromolecules as well as cell surface adhesion molecules. At this juncture, we posit that changes in the dynamics of ECM turnover and cell surface molecule expression occurring within the vascular wall lead to the unregulated proliferation of VSMCs and the development of a pro-inflammatory phenotype. Interestingly, at least in terms of proliferative responses, the use of a 3-D spheroid model allowed us to recapitulate the MT1-MMP-dependent biological processes *ex vivo*. Nevertheless, it remains unclear how cleavage of one or more MT1-MMP substrates accelerates VSMC proliferation in APOE-null mice. This caveat notwithstanding, our data clearly highlight the role played by VSMC-derived MT1-MMP in limiting the progression of proliferative atherosclerotic lesions. As MT1-MMP is a membrane-bound proteinase, the physical proximity of substrates with the enzyme is likely critical for the protective effects exerted by VSMCs in limiting the expansion of atherosclerotic lesions. Our study also suggests that other atherosclerosis-associated MMPs, e.g., MMP-2, MMP-8, MMP-13, do not compensate for the genetic loss of MT1-MMP and are unable to limit atherosclerosis progression. While MT2-MMP (MMP15) was also expressed in mouse VSMCs (Fig. S2), as reported previously in rat VSMCs [41], the biological phenotypes conferred by MT1-MMP gene loss were not rescued by the presence of MT2-MMP. Differences in the hemopexin domain structure, posttranslational modification, or protein trafficking may underlie the specific effects mediated by MT1-MMP versus MT2-MMP [42].

While increased collagen content within atheroma might be predicted to play a protective role against plaque rupture, collagen accumulation in arterial walls could also accentuate vascular sclerosis and stiffening. In turn, stiff and sclerotic blood vessels could increase luminal sheer stress, thereby increasing the chance of plaque rupture. In future studies, an assessment of hemodynamic changes and plaque instability in our model will be required to more accurately define the role of MT1-MMP in cardiovascular disease. Interestingly, in APOE-null mice, arterial wall stiffness is known to be increased through VSMC lysyl oxidase activity [43]. In turn, increased tissue stiffness could control VSMC proliferation through the activation of a mechanotransduction pathway, e.g., YAP/TAZ transcription activity [44]. As such, we posit that the

pathologic accumulation of ECM macromolecules secondary to the loss of MT1-MMP activity may further promote vascular wall rigidity in the APOE-null mice, leading to hyper-proliferative vascular lesions and aneurysm formation. Given that we observed severe atherosclerosis and aneurysm formation in iliac and femoral arteries, site-specific increases in vascular wall thickening and arteriosclerosis are likely related to the distinct mechanical properties of vessel walls observed along the arterial tree [45]. Indeed, femoral arteries as well as the abdominal aorta are known to display higher wall thickness with lower content of elastic lamina compared to carotid arteries and thoracic aortas [45]. Together, differences in mechanical stress and ECM composition may render VSMCs in abdominal aortae and femoral arteries more vulnerable to atherogenic proliferation.

Finally, it remains to be determined whether the inflammatory gene expression profile observed in MT1-MMP-null VSMCs is restricted to atherogenic milieu encountered *in vivo*. Interestingly, in our hands, the increase in inflammatory gene expression of MT1-MMP-null VSMCs was coupled with decreased mitochondrial activity (unpublished data). Further, recent studies have demonstrated a critical role played by a VSMC phenotypic switch to macrophage-like cells in atherogenesis [4, 46]. As MT1-MMP also regulates inflammatory responses in macrophages [47], the proteinase may well play a key role in controlling a complex set of metabolic and phenotypic switching programs that are engaged in atherogenic VSMCs. While further work is needed to delineate MT1-MMP function during atherogenesis, our work highlights the previously unsuspected vessel wall-protective effects exerted by this membrane-anchored metalloproteinase in the VSMC compartment.

Acknowledgements: We like to thank Dr. Pierre Chambon (Institute of Genetics and Molecular and Cellular Biology, France) for sharing *Fabp4-Cre-ERT2* transgenic mice. Current affiliation of Miina K. Öhman is Duke-Nus Medical School, Singapore.

Funding Sources: Funding was provided by the McKay Research Grant from University of Michigan Cardiovascular Center, NIH R21HL106332, and R01DK102656 to Tae-Hwa Chun. NIH R01AI105068-01 to Stephen J. Weiss. NIH Cancer Biology Training Program Grant T32-CA009676 supported Julian Bahr.

Disclosures: None.

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Figure Legends:

Figure 1. MT1-MMP heterozygosity promotes atherosclerosis. (A) *Mmp14*$^{+/+}$*Apoe*$^{-/-}$ mice and *Mmp14*$^{+/-}$*Apoe*$^{-/-}$ mice were fed a Western diet for 12 weeks beginning at 8 weeks of age. Body weight (g) at the end of study. (B) Percent fat mass (wt/wt) of total (epididymal + inguinal) and epididymal fat pads. (C) Fasting serum cholesterol (total and direct LDL) levels. Mean ± SEM, n = 8 and 10, respectively, *, $P < 0.05$ (D) Atherosclerotic lesions detected with Oil-Red-O staining in *Mmp14*$^{+/+}$*Apoe*$^{-/-}$and *Mmp14*$^{+/-}$*Apoe*$^{-/-}$ male mice. Arrows point to the increased atherogenesis distributed in abdominal aorta and iliac arteries specifically, as found in *Mmp14*$^{+/-}$ *Apoe*$^{-/-}$ mice. (E, F) Oil-Red-O-positive atherosclerotic plaque areas quantified in all aortic trees and abdominal aortas in male mice (n = 7 each) and female mice (n = 4 and 10). Mean ± SEM, * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$

Figure 2. MT1-MMP heterozygosity promotes plaque formation and outward vascular remodeling. (A) Representative histology sections of left common carotid arteries. H&E and Sirius Red staining. Scale = 100 µm. (B) Sirius Red-positive area (%). (C) Immunostaining of smooth muscle actin (SMA) in vascular walls and atherosclerotic plaques (orange). Nuclei were counterstained (blue). Lower panels are of higher magnification. Scale = 100 µm. (D) SMA-positive area (%).

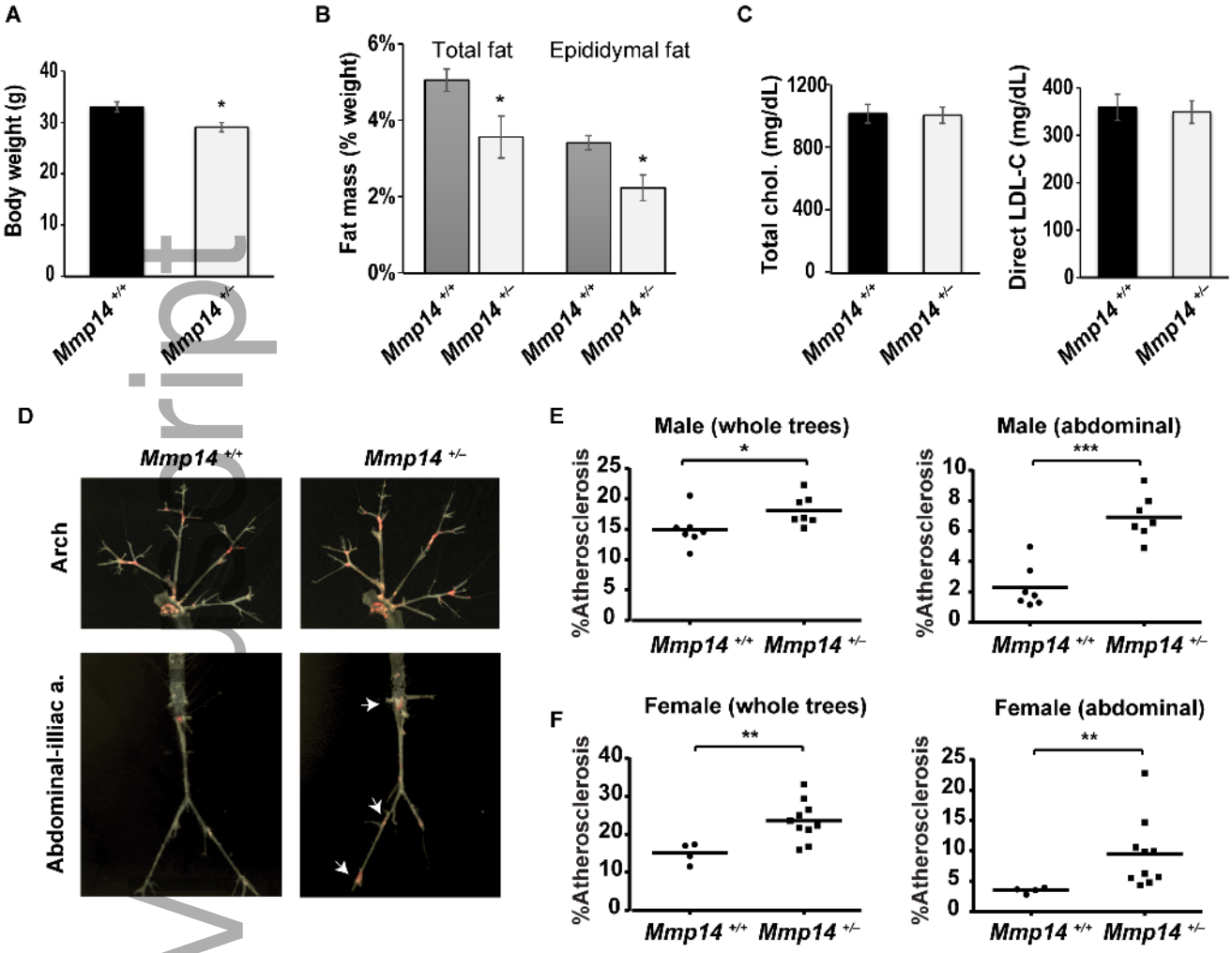
Figure 3. VSMC MT1-MMP gene targeting promotes atherosclerosis and aneurysm formation. (A) Whole arterial trees assessed for Oil-Red-O-positive atherosclerotic plaque area (red). Arrows point to atherosclerotic aneurysms. Representative arterial trees from SM22α-Cre(–)*MMP14*$^{F/F}$*Apoe*$^{-/-}$ and SM22α-Cre(+)*MMP14*$^{F/F}$*Apoe*$^{-/-}$ mice are shown. N=13 and 20, respectively. (B) Higher magnification of aortic aneurysms found in SM22α-Cre(+)*MMP14*$^{F/F}$*Apoe*$^{-/-}$ mice. (C) Percent plaque area in *Cre(–)Mmp14*$^{F/F}$*Apoe*$^{-/-}$, *SM22α-Cre(+)Mmp14*$^{F/F}$*Apoe*$^{-/-}$*, aP2-Cre-ERT2(+)Mmp14*$^{F/F}$*Apoe*$^{-/-}$ mice, males and females (n = 8, 13, 4 in male mice and n = 5, 7, 7 in female mice, respectively). * $P < 0.05$, *** $P < 0.0005$.

Figure 4. The loss of vascular wall integrity and VSMC proliferation in VSMC MT1-MMP KO mice. (A) Atherosclerotic aneurysms found in iliac arteries of SM22α-Cre(+)*MMP14*$^{F/F}$*Apoe*$^{-}$

$^{/-}$ mice. Masson Trichrome staining; the border between the vascular wall and the atheroma is demarcated with a dashed line. Verhoeff-Van Gieson staining (VVG), SMA staining, Ki67 staining, and F4/80, a macrophage marker, staining. Scale = 100 μm. The lower magnification (4x) micrograph of SM22α-Cre(+)*MMP14$^{F/F}$Apoe$^{-/-}$* VVG staining on the right. Scale = 200 μm. The lesion in the dashed square is shown on the left. (B) Plaque area. (C) The internal diameter of iliac arteries. (D) Ki67-positive area. Mean ± SEM. n = 5 and 7 for each group. One-way ANOVA. *P < 0.05, **P < 0.005

Figure 5. Proinflammatory gene expression in MT1-MMP-null VSMCs. (A) Primary aortic VSMCs isolated from *Cre(–)Mmp14$^{F/F}$Apoe$^{-/-}$* and *SM22α-Cre(+)/Mmp14$^{F/F}$Apoe$^{-/-}$* mice. F-actin (green), nuclei (blue). (B) Representative genes differentially expressed in VSMCs from two independent pairs of *Cre(–)Mmp14$^{F/F}$Apoe$^{-/-}$* and *SM22α-Cre(+)/Mmp14$^{F/F}$Apoe$^{-/-}$* mice. (C) Gene Ontology-Biological Processes represented by the genes upregulated in VSMCs isolated from the *SM22α-Cre(+)/Mmp14$^{F/F}$Apoe$^{-/-}$* mice.

Figure 6. MT1-MMP limits VSMC proliferation in 3-D organoids. (A) Primary VSMCs isolated from Cre(-)*Mmp14$^{F/F}$Apoe$^{-/-}$* mice and Cre(+)*Mmp14$^{F/F}$Apoe$^{-/-}$* mice were cultured as a 3-D spheroids for 48 hours and stained for Ki67 (red), nuclei (DAPI, blue), and actin (phalloidin, green). (B) Quantified intensity of Ki67 staining per spheroid. n = 5~7. * P < 0.05 (C) Immortalized mouse VSMCs (MOVAS) transiently transfected with siRNA control (siControl) and MT1-MMP siRNA (si*Mmp14*). Ki67 (red), nuclei (blue), actin (green). (D) Ki67 staining intensity per spheroid of MOVAS. ** P < 0.005 (E) 2-D cultured MOVAS transfected with control and MT1-MMP siRNA. Ki67 (red), nucleus (blue), and actin (green). (F) Ki67-positive nuclei per total nuclei count.



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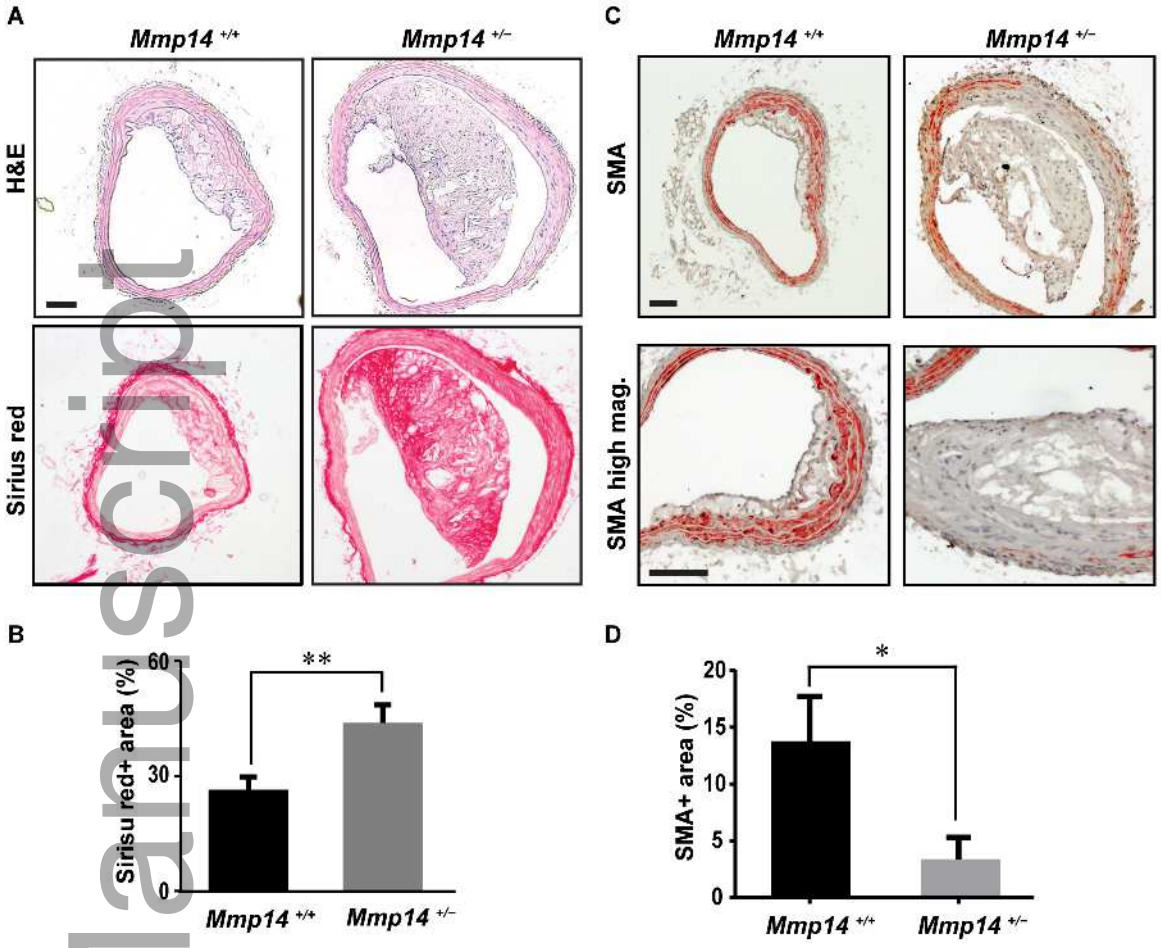

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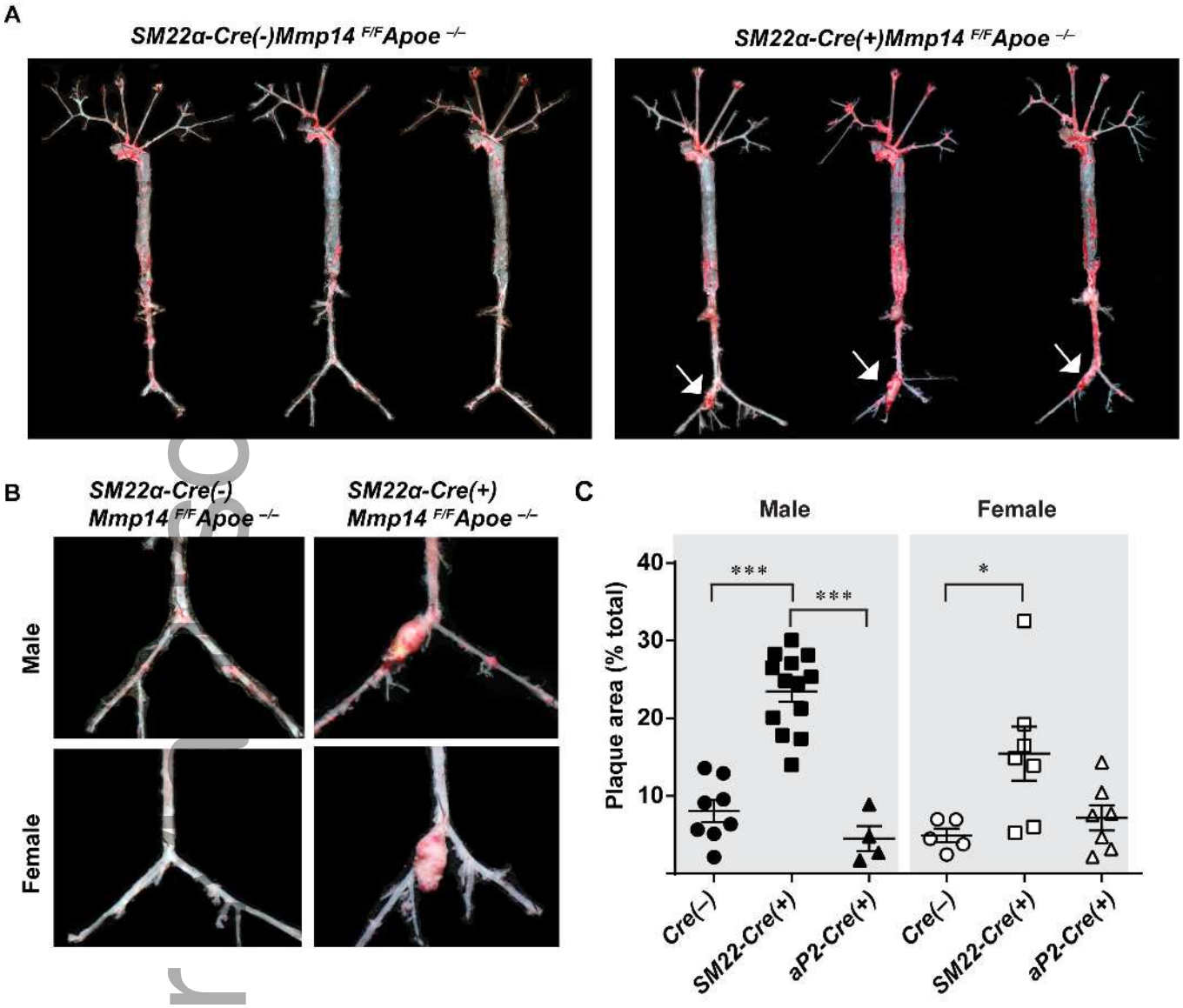

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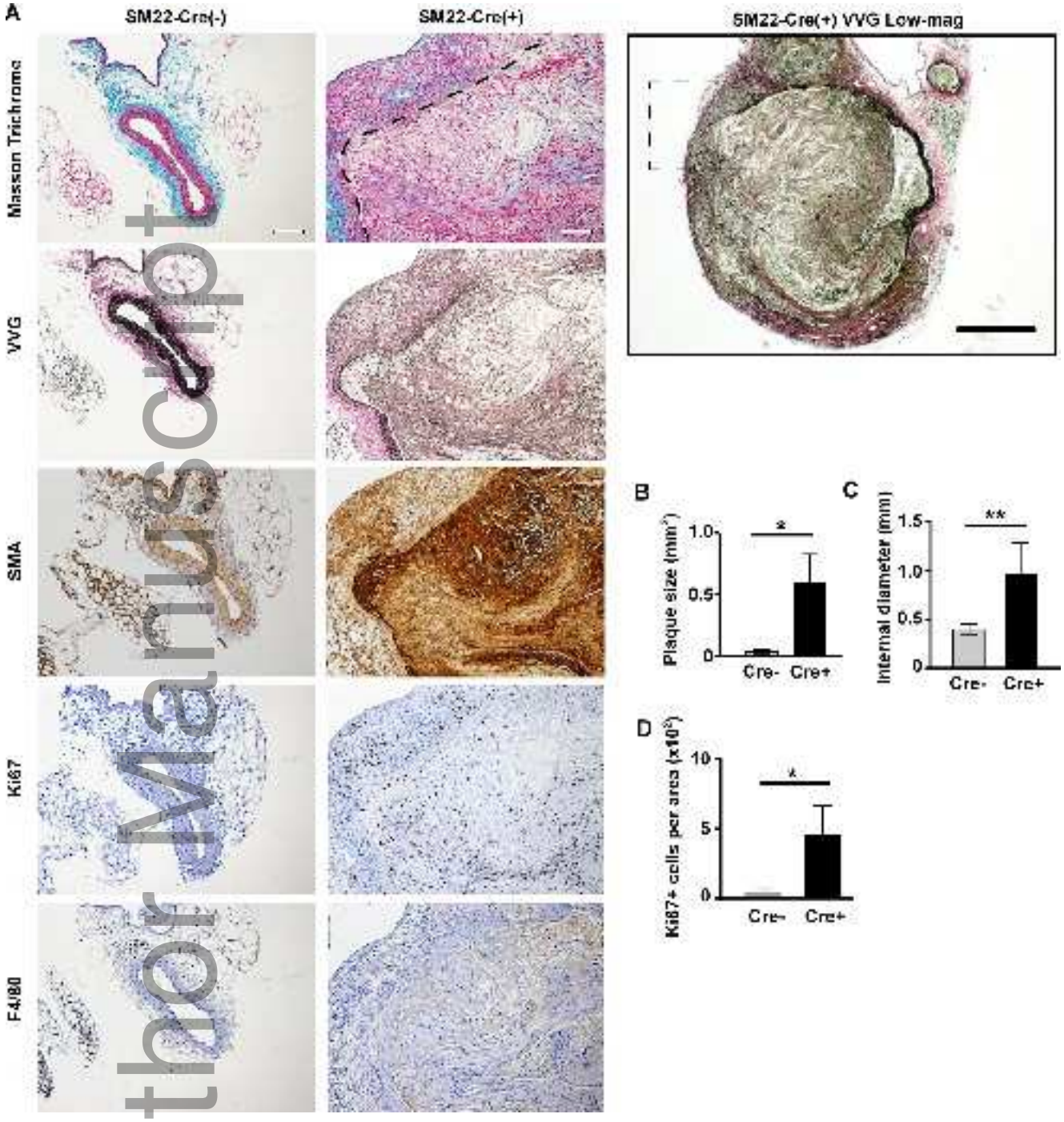

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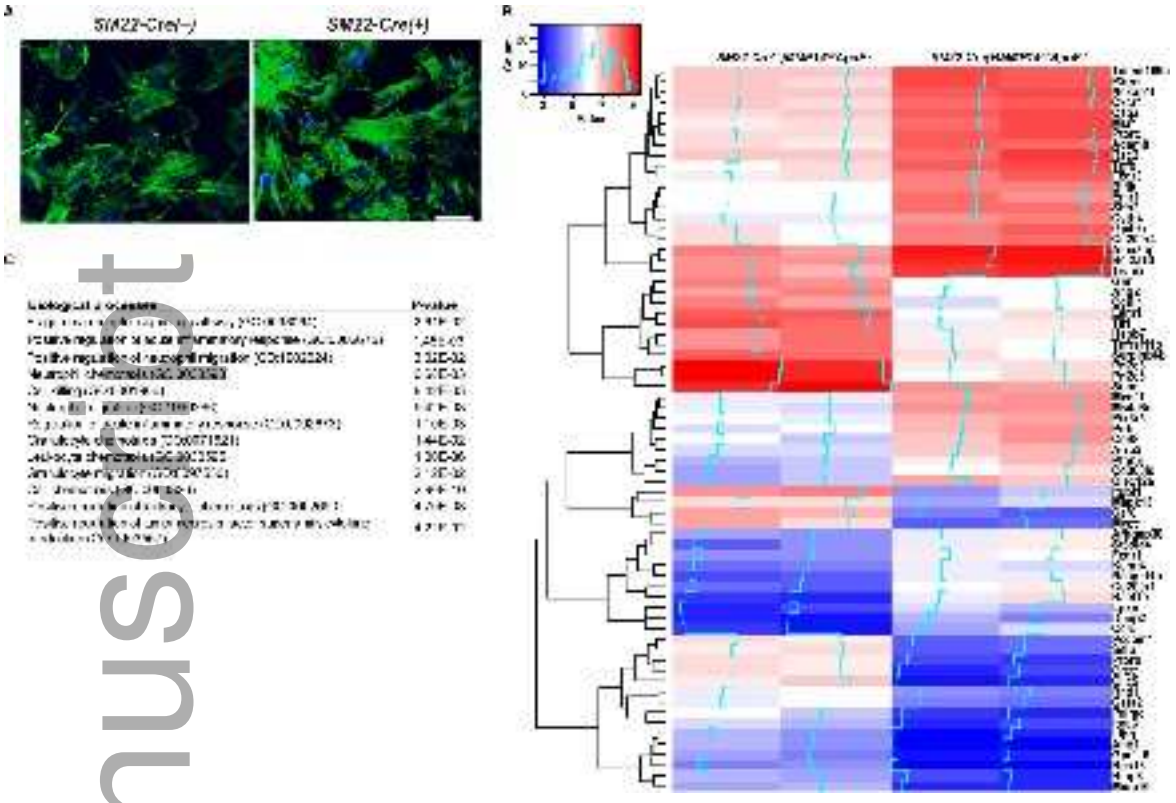

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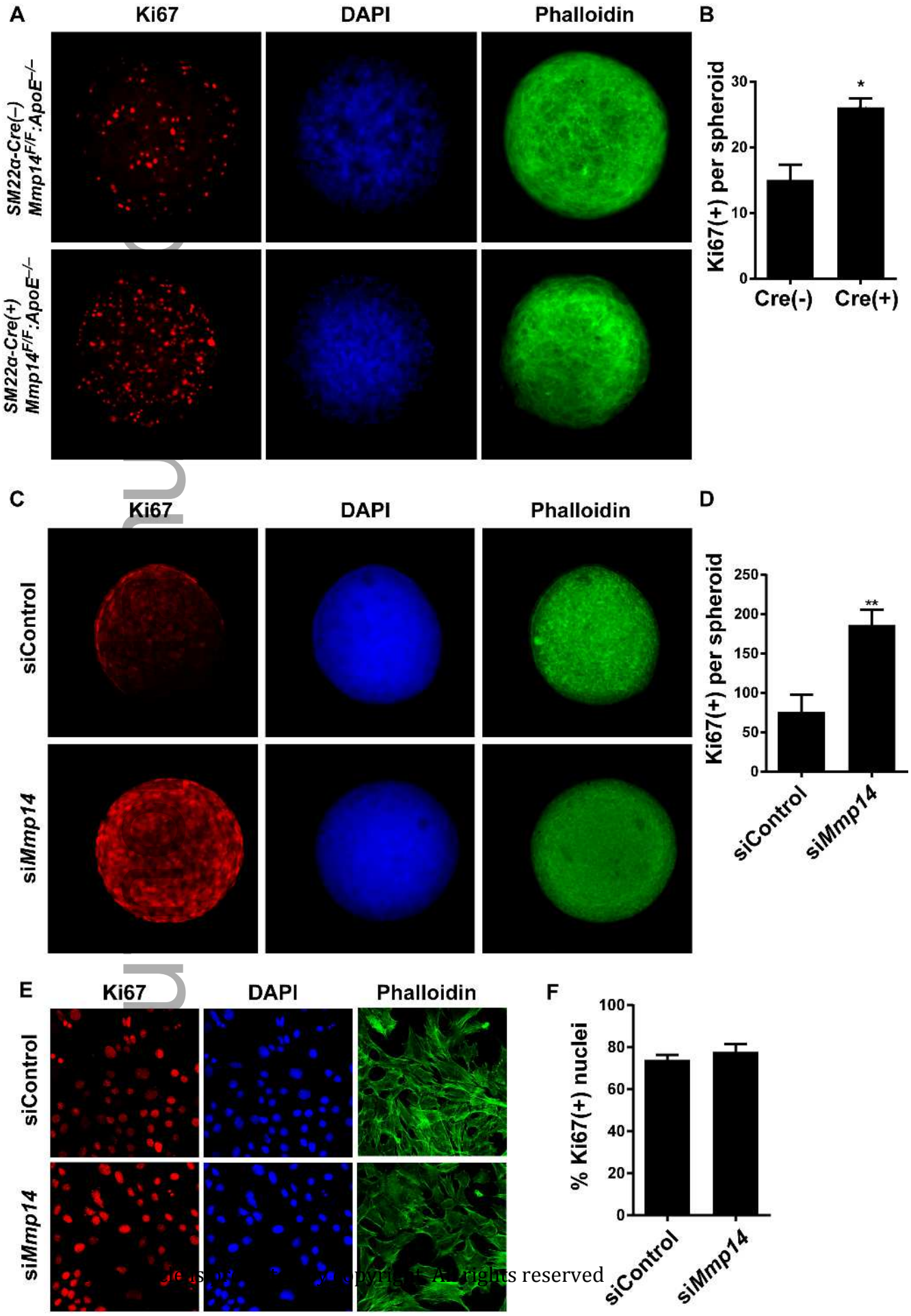

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