活習慣病対策の食事指導を頑なに守り続けていることがある．加齢とともに徐々に体重が減少し，低栄養に傾きやすい．油は高齢者にとって効率のよいカロリー供給源である．肉も重要な蛋白供給源である．かかりつけ医も若年者と同様の画一的な生活習慣病に対する指導を考え直すべきである．もちろん，後期高齢者といえども，虚血性心疾患，糖尿病の存在，また関節症などの存在のため体重管理や厳格な食事療法(カロリー制限，動物性脂肪の制限)が必要な場合がある．しかし，それ以外の場合無理な減量，食事療法によりかえって栄養状態を悪化させることもあり，画一的な指導には十分注意する必要がある．

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Exercise Training Stimulates Ischemia-Induced Neovascularization via Phosphatidylinositol 3-Kinase/Akt-Dependent Hypoxia-Induced Factor-1α Reactivation in Mice of Advanced Age

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Background—Exercise stimulates the vascular response in pathological conditions, including ischemia; however, the molecular mechanisms by which exercise improves the impaired hypoxia-induced factor (HIF)-1α–mediated response to hypoxia associated with aging are poorly understood. Here, we report that swimming training (ST) modulates the vascular response to ischemia in aged (24-month-old) mice.

Methods and Results—Aged wild-type mice (MMP-$2^{+/+}$) that maintained ST (swimming 1 h/d) from day 1 after surgery were randomly assigned to 4 groups that were treated with either vehicle, LY294002, or deferoxamine for 14 days. Mice that were maintained in a sedentary condition served as controls. ST increased blood flow, capillary density, and levels of p-Akt, HIF-1α, vascular endothelial growth factor, Fit-1, and matrix metalloproteinase-2 (MMP-2) in MMP-$2^{+/+}$ mice. ST also increased the numbers of circulating endothelial progenitor cells and their function associated with activation of HIF-1α. All of these effects were diminished by LY294002, an inhibitor of phosphatidylinositol 3-kinase; enhanced by deferoxamine, an HIF-1α stabilizer; and impaired by knockout of MMP-2. Finally, bone marrow transplantation confirmed that ST enhanced endothelial progenitor cell homing to ischemic sites in aged mice.

Conclusions—ST can improve neovascularization in response to hypoxia via a phosphatidylinositol 3-kinase–dependent mechanism that is mediated by the HIF-1α/vascular endothelial growth factor/MMP-2 pathway in advanced age. (***Circulation.*** **2010;122:707-716.)**

Key Words: exercise ■ angiogenesis, physiological ■ phosphatidylinositol 3-kinase ■ hypoxia-inducible factor 1, α subunit ■ aging ■ neovascularization, physiological

Aging is associated with a decreased ability to form new blood vessels in response to ischemia, which results in higher rates of cardiovascular complications and diminished capacity for tissue regeneration.[1] There is therefore considerable interest in understanding the mechanisms of angiogenesis in advanced age. Accumulating evidence suggests that the process of new blood vessel formation is associated with extracellular matrix remodeling, mainly involving the matrix metalloproteinase (MMP) family.[2,3] In particular, aging reduces MMP-2 expression in vitro and in vivo.[4,5] Genetic and pharmacological intervention studies have demonstrated in several animal models that MMP-2 plays an important role in angiogenesis and vasculogenesis.[5,6] Recently, a few studies have shown that knee-extension exercise activates MMP expression in human skeletal muscle.[7] On the basis of these findings and past reports that exercise increases coronary vascularization by promoting vascular growth and remodeling in response to stress,[8] we hypothesize that the activation of MMP-2 might represent a crucial mediator by which exercise triggers protective vascular action.

Clinical Perspective on p 716

Administration of bone marrow (BM)–derived or peripheral blood–derived endothelial progenitor cells (EPCs) has improved postischemic neovascularization in various experimental and clinical trials[9,10]; however, several recent randomized clinical

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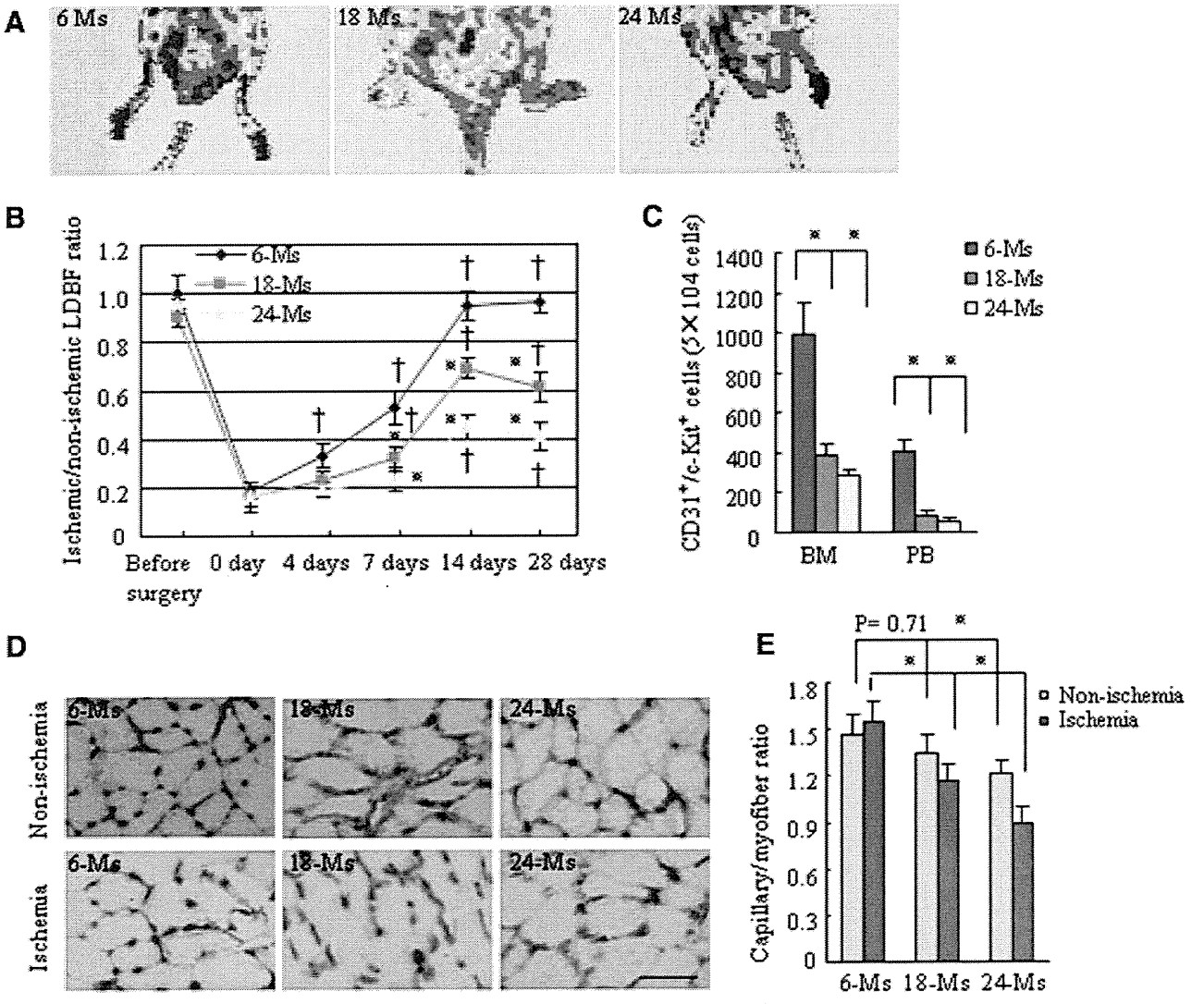

Figure 1. Aging reduces vessel density and blood flow in ischemic tissues of MMP-$2^{+/+}$ mice. A, A low perfusion signal (dark blue) was observed in the ischemic hindlimbs of aged MMP-$2^{+/+}$ mice (18 and 24 months old) with laser Doppler perfusion imaging, whereas a high signal (red) was detected in young (6-month-old) MMP-$2^{+/+}$ mice. B, The ratio of ischemic-to-normal laser Doppler blood flow (LDBF) in aged MMP-$2^{+/+}$ mice (n=10 per group; †$P<0.05$ vs each group at day 0, *$P<0.05$ vs the corresponding 6-month-old mice at days 7 to 28 after ischemia; 2-way repeated-measures ANOVA and Bonferroni post hoc tests). C, Quantitative analysis of the numbers of EPCs in BM and peripheral blood (PB) of WT mice (n=10 per group; *$P<0.05$, Tukey post hoc test). D, Immunohistostaining showed the capillaries in the thigh adductor muscle at postoperative day 28. Scale bar=100 μm. E, Quantitative analysis of capillary density in 3 groups of mice (n=8 per group; *$P<0.05$, paired Student *t* test). Ms indicates months.

trials of stem and progenitor cell treatment for ischemic diseases have been disappointing in subjects of advanced age.[11] Impaired angiogenesis in advanced age might be due to an intrinsic decline in the regenerative capacity of vascular progenitors or a decline in a proregenerative niche.[12] On the other hand, physical training increases circulating EPCs in patients with ischemic syndromes.[13] Further work is necessary to determine whether exercise improves EPC mobilization and function in individuals of advanced age, as well as to determine the mechanisms underlying these processes.

Exercise promotes ischemic angiogenesis by increasing vascular endothelial growth factor (VEGF) in plasma or ischemic tissue in humans and animals[14,15]; however, angiogenic growth factors and related transcriptional factor hypoxia-induced factor-1α (HIF-1α) activity decreased in aged cell lines and animals.[16,17] In the present study, we investigate the effects of swimming training (ST) on angiogenic mechanisms and HIF-1α function in a mouse model of limb ischemia at advanced age. We evaluated whether ST was able (1) to enhance HIF-1α transcriptional activity through activation of the insulin-like growth factor (IGF)-1–mediated phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway and attenuation of the prolyl hydroxylases (PHDs) degradation system, (2) to stimulate reactivation of VEGF and MMP-2 expression in ischemic tissue and BM-derived EPCs, (3) to improve EPC mobilization and homing to the vasculature, and (4) to enhance neovascularization in response to hypoxia.

Methods

An expanded Methods section is available in the online-only Data Supplement.

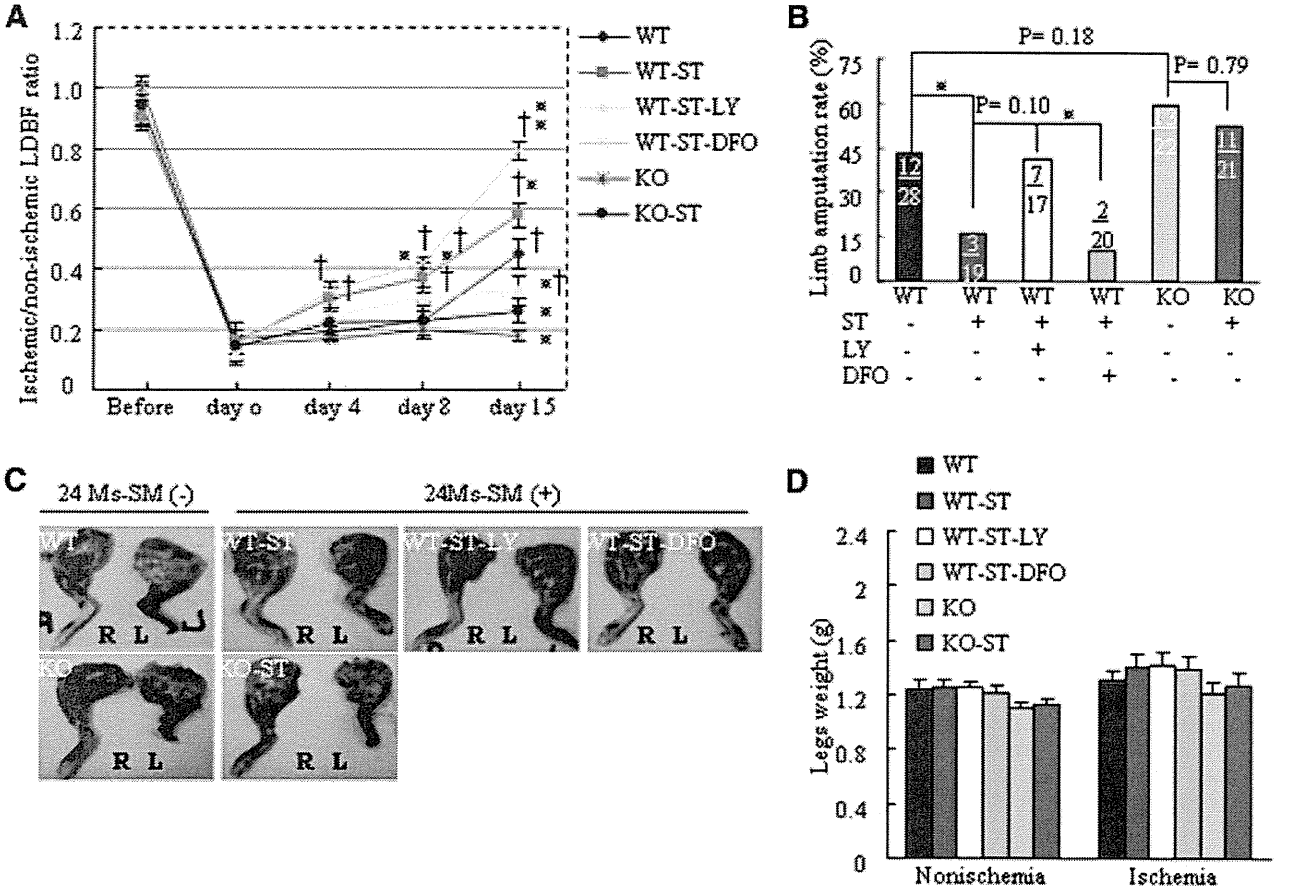

Figure 2. ST restores ischemic revascularization in angiogenesis-defective 24-month-old mice by postoperative day 15. A, The ratio of ischemic-to-normal laser Doppler blood flow (LDBF) in aged MMP-$2^{+/+}$ mice (n=8 per group; †$P<0.05$ vs corresponding day 0, *$P<0.05$ vs corresponding MMP-$2^{+/+}$ mice during ischemia; 2-way repeated-measures ANOVA and Bonferroni post hoc tests). B, Quantitative analysis of foot amputation in 6 groups (*$P<0.05$, χ^2 test). Upper number indicates number of amputations; lower number, number of animals. C, Photographs of typical hindlimbs of the 6 groups of mice. R indicates right (nonischemic); L, left (ischemic). D, Weights of ischemic and nonischemic legs of mice (paired Student *t* test). Ms indicates months.

Mouse Model of Revascularization Without or With Exercise

Studies of wild-type (WT; MMP-$2^{+/+}$; Chubu Kagaku Shizai Co., Ltd. Nagoya, Japan) and MMP-2 knockout (KO, MMP$^{-/-}$, gifted by S. Itohara RIKEN Brain Science, Institute, Wako, Saitama, Japan)[5] mice in a C57/BL6 background were approved by the Animal Studies Committee of Nagoya University. Male young (6 months) and aged (18 and 24 months) mice of both genotypes were subjected to unilateral hindlimb ischemic surgery and ST programs.

Statistical Analysis

Data are expressed as mean±standard error of the mean (SEM). Student *t* tests (for comparison between 2 groups) or 1-way ANOVA (for comparison of 3 or more groups) followed by Tukey post hoc tests were used for statistical analyses. The nonparametric Kruskal-Wallis test (Tukey-type multiple comparison) was used ANOVA for the gene expression data. Blood flow data were subjected to 2-way repeated-measures ANOVA and Bonferroni post hoc tests. The comparative incidence of limb amputation was evaluated by the χ^2 test. SPSS software version 17.0 (SPSS Inc, Chicago, Ill) was used. A value of $P<0.05$ was considered statistically significant.

Results

Aging Reduces HIF-1α–Induced Growth Factors and Impairs Neovascularization in Response to Hypoxia

Serial laser Doppler blood flow measurements showed that aged WT mice (18 to 24 months old) had lower ratios of ischemic-to-nonischemic blood flow (Figure 1A and 1B) than young (6-month-old) WT mice. The ratio decreased further from 18 to 24 months of age. The numbers of CD31$^+$/c-Kit$^+$ progenitor cells in both BM and peripheral blood also decreased markedly in an age-dependent manner (Figure 1C), which suggests a vasculogenesis-specific impairment with age. The capillary density of nonischemic and ischemic muscle also correlated with age (Figure 1D and 1E).

ST Restores Ischemic Neovascularization in Mice of Advanced Age (24 Months)

On day 15 after the induction of ischemia, aged WT mice exposed to ST (WT-ST mice) had markedly higher blood perfusion than WT mice (Figure 2A), which suggests that ST stimulated neovascularization in response to hypoxia. This was further supported by data from longer-term ST (29 days; online-only Data Supplement Figure I). ST also increased capillary density (Figure 3A). HIF-1α, which is regulated by the PI3K signaling pathway, is less stable and active in aged animals during ischemia.[18,19] We hypothesized that ST protects against HIF-1α destabilization by activating the PI3K signaling pathway; the increased stability of HIF-1α would then increase ischemic neovascularization. We tested this hypothesis by treating aged WT mice with LY2940029 (LY), an inhibitor of PI3K,

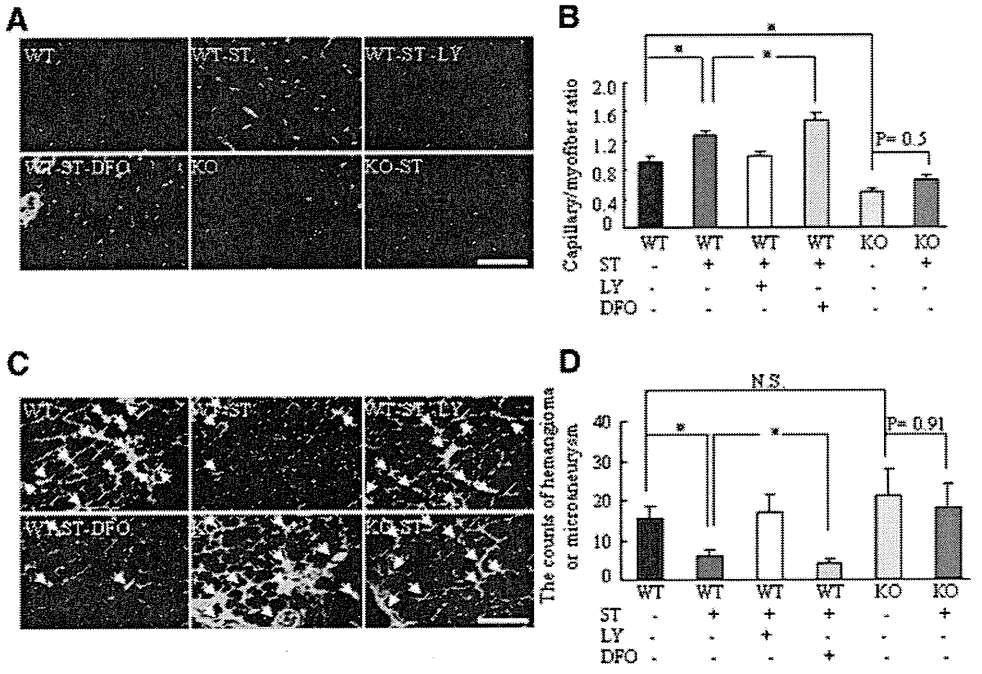

Figure 3. ST promotes ischemic revascularization and reduces pathological vascularity in 24-month-old mice by postoperative day 15. A, Immunohistostaining showing the capillary density of ischemic muscle. Scale bar=100 μm. B, Quantitative analysis of capillary density in the 6 experimental groups. (n=6 per group; *$P<0.05$, Tukey post hoc test). C, Immunofluorescence of the ischemic leg vasculature. Scale bar=100 μm. D, Quantitative analysis of the numbers of aberrant vessels, such as hemangiomas or microaneurysms, in the 6 experimental groups (n=6 per group; *$P<0.05$, Tukey post hoc test).

or deferoxamine (DFO). ST-mediated improvement of blood perfusion and capillary density were diminished by use of LY in WT-ST mice; these beneficial effects of ST treatment were enhanced by DFO (Figures 2A, 3A, and 3B).

Aged MMP-$2^{-/-}$ mice exhibited a marked impairment of blood perfusion and capillary density after hindlimb ischemia compared with MMP-$2^{+/+}$ mice; these changes were not improved by ST treatment (Figures 2A, 3B, and 3B). This finding supports the notion that ST stimulates an angiogenic response via MMP-2 activation in ischemic tissue of aged animals. Furthermore, spontaneous amputation occurred in 42.9% of the ischemic limbs of WT mice and in 15.7% of ischemic limbs of WT-ST mice. The beneficial effect of ST was diminished in WT-ST-LY mice (41.2% of limbs) and was slightly enhanced in WT-ST-DFO mice (10% of limbs; Figure 2B). The rate of spontaneous amputation in KO mice (59.1%) was slightly higher than in WT mice and was not affected by ST (52.3%). At day 15 after surgery, there were no significant differences between the weights of nonischemic and ischemic legs among the 6 experimental groups of mice (Figures 2C and 2D). Additionally, DFO alone increased blood perfusion and capillary density in aged mice without ST, whereas LT alone did not have these effects (online-only Data Supplement Figure II).

ST Prevents the Formation of Pathological Vessels and Restores Collateral Artery Formation in Response to Hypoxia in Mice at 24 Months

Abnormal corkscrewlike vessels with irregular lumens were visible in aged mice (Figure 3C). Some vessels were dilated and formed microaneurysms and hemangiomas. In contrast, fewer pathological vessels were observed in WT-ST and WT-ST-DFO mice than in WT mice; this effect was diminished in WT-ST-LY mice (Figure 3D). On postoperative day 15, the microangiography and angiographic score revealed that the ischemic hindlimbs of 24-month-old WT-ST mice had well-developed collateral arteries, and the numbers of collateral vessels (vessel diameter >0.30 μm, of non-ST control) was improved significantly by ST, resulting in an angiographic score 135% of that in WT-ST mice; this effect was attenuated to a score of 98% in ST-LY mice and enhanced to a score 178% of ST-DFO mice (Figure 4A and 4B). Similarly, fluorescent staining with α-smooth muscle actin showed that the ischemic-to-nonischemic ratio in mature neovessels was higher in WT-ST mice than in WT mice; this change was attenuated by LY and enhanced by DFO (Figures 4C and 4D). These findings suggest that ST might promote collateral vessel formation and maturation and prevent pathological vessel formation via PI3K-dependent HIF-1α activation and stabilization, which influences ischemic tissue blood perfusion. However, ST had no significant effect on the formation of collateral and pathological vessels in aged MMP-$2^{-/-}$ mice.

ST Enhances the Hypoxia Response Through Stimulation of the PI3K Signaling Pathway and Prevention of HIF-1α Destabilization in 24-Month-Old Mice

Western blot analysis revealed that the levels of p-Akt and HIF-1α were higher in WT-ST mice than in WT mice; these effects were diminished by LY (Figures 5A and 5E). We then evaluated whether ST prevents age-mediated HIF-1α destabilization. ST reduced the levels of PHD-3 and factor inhibiting HIF-1 (FIH) proteins from those in aged non-ST-WT mice (Figures 5A and 5D). Levels of VEGF and Flt-1 mRNAs, as well as of stromal cell–derived factor-1, were higher in WT-ST

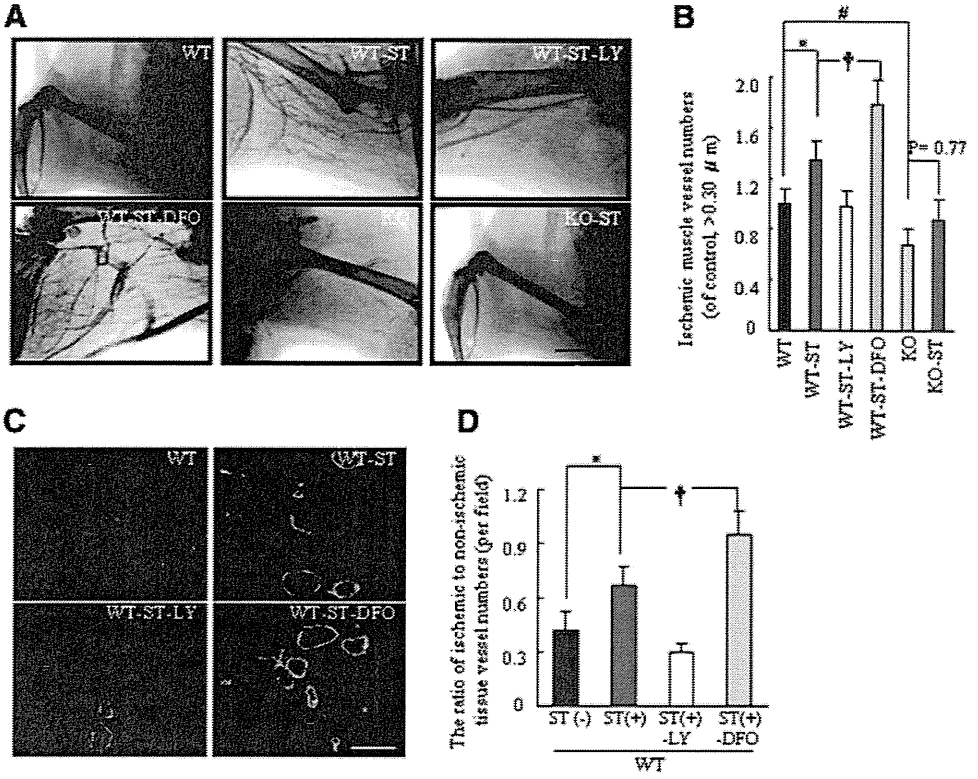

Figure 4. ST stimulates collateral artery formation in response to hypoxia in 24-month-old MMP-$2^{+/+}$ mice by postoperative day 15. A, Representative microangiography showing collateral artery formation of ischemic tissues of the 6 experimental groups. B, Quantitative analysis of microangiography showed the numbers of neovessels (>0.30 μm per field) in ischemic muscles of the 6 groups (n=4 per group; *P<0.05, †P<0.05, #P<0.05, Tukey post hoc tests). C, Fluorescence staining of ischemic tissue of the 4 indicated MMP-$2^{+/+}$ experimental groups with mouse anti-human α-smooth muscle actin. D, Quantitative analysis of the ratio of neovessels in ischemic to nonischemic tissue in the 4 groups (n=6 per group; *P<0.05, †P<0.05, Tukey post hoc tests).

mice than in WT mice; these effects were diminished by LY and enhanced by DFO (Figure 5A and 5C; online-only Data Supplement Figure III). Similarly, the level of MMP-2 mRNA was also higher in WT-ST mice than in WT mice; this effect was diminished by LY and enhanced by DFO (Figure 5A and 5B). In addition, ST enhanced the IGF-1 protein level in ischemic muscle of aged mice of both genotypes (Figure 5A and 5E). VEGF has been shown to regulate MMP-2 expression in several cell lines.[5] Taken together, these findings suggest that ST likely provides these beneficial effects on ischemic revascularization in aged mice through VEGF/Flt-1–mediated MMP-2 activation induced by stimulation of IGF-1/PI3K/Akt-dependent HIF-1α synthesis and prevention of HIF-1α destabilization. On the other hand, the level of VEGF mRNA was lower in the ischemic muscle of aged MMP-$2^{-/-}$ mice than in aged MMP-$2^{+/+}$ mice. Immunostaining demonstrated that macrophage infiltration was impaired in the ischemic tissues of aged MMP-$2^{-/-}$ mice (online-only Data Supplement Figure IV). A previous study demonstrated that the reduction of VEGF in the plasma and ischemic tissues of young MMP-$2^{-/-}$ mice results from decreased inflammatory cell infiltration.[5] On the basis of these findings, we can conclude that MMP-$2^{-/-}$–mediated impairment of macrophage infiltration may also contribute to the decrease in the level of VEGF in aged mice.

To test this hypothesis, we examined the ability of ST to activate the PI3K/HIF-1α/VEGF signaling pathway in aged MMP-$2^{-/-}$ mice. The increase in levels of p-Akt, HIF-1α, VEGF, and Flt-1 in aged MMP-$2^{-/-}$-ST mice (Figure 5A, 5C, 5D, and 5E) supports the notion that activation of the PI3K/HIF-1α/VEGF/MMP-2–dependent angiogenic pathway might represent a critical molecular mechanism by which ST triggers angiogenesis. The absence of the last step in this pathway (MMP-2) prevents the functional effects of activating this pathway. Otherwise, it appears that the ability of this pathway to be activated in KO mice actually does not support a role for the pathway. There were no significant differences in the levels of PHD-1 and total Akt proteins among the 6 experimental groups.

ST Stimulates EPC Mobilization and Improves the Intrinsic Function of EPCs in 24-Month-Old Mice

At day 7 after ST, the numbers of $CD31^{+}$/c-Kit^{+} EPC-like cells were higher in WT-ST mice than in aged WT mice as measured by flow cytometry; this effect was diminished by LY and enhanced by DFO (Figure 6D). The levels of HIF-1α protein and MMP-2 mRNA in the EPCs from WT-ST mice were higher than in those from WT mice; these changes were diminished by LY and enhanced by DFO (Figure 6A through 6C). This finding led us to hypothesize that ST-mediated activation of HIF-1α and MMP-2 may facilitate the mobilization of EPC-like cells from the BM into the circulation to support revascularization in older mice. The numbers of adherent and invading EPCs were higher in WT-ST mice than in WT mice (Figure 6E and 6F). Strikingly, the tubulogenic response was also better in EPCs derived from WT-ST mice

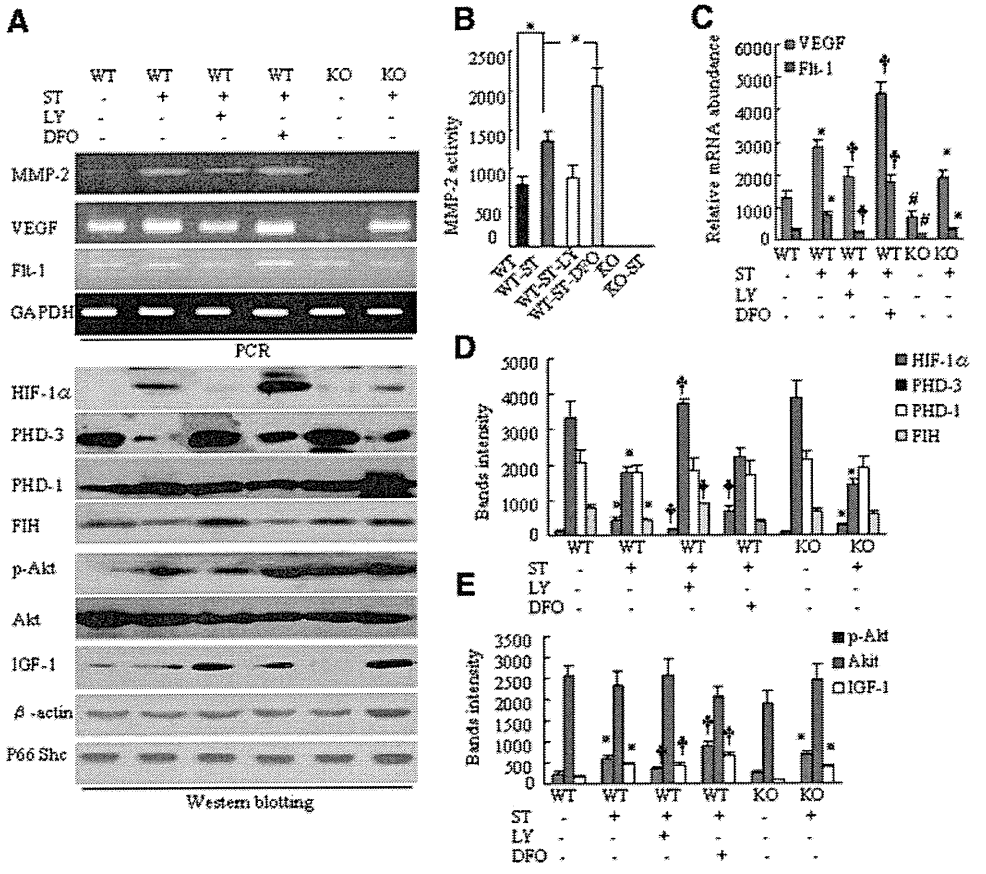

Figure 5. Expression of targeted genes in the 6 groups of 24-month-old mice on postoperative day 8. A, Representative polymerase chain reaction (PCR) and immunoblots show the levels of the targeted gene or protein in the ischemic muscles of mice in the 6 experimental groups. B, Quantitative analysis of gelatin zymography for MMP-2 gelatinolytic activity in the 6 groups (n=4 per group; *$P<0.05$). C, Quantitative analysis of PCR bands for levels of VEGF and Flt-1 mRNAs in the 6 groups (n=4 per group; *$P<0.05$, †$P<0.05$). D and E, Quantitative analysis of Western blots for levels of HIF-1α, p-Akt, IGF-1, PHD-3, FIH, total Akt, and PHD-1 proteins in the 6 groups (n=4 per group). Expression levels of targeted genes were normalized with a PCR or Western blot with primer or antibodies, respectively, to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin, or Shc. Kruskal-Wallis tests and Tukey post hoc tests were each used for statistical analyses. *$P<0.05$ vs corresponding MMP-$2^{+/+}$ or MMP-$2^{-/-}$, †$P<0.05$ vs MMP-$2^{+/+}$-ST, #$P<0.05$ vs MMP-$2^{+/+}$.

than in those derived from WT mice (Figure 6E and 6H). These findings suggest that an improvement of EPC cellular function may contribute, in part or entirely, to the restored revascularization seen with ST in advanced age.

ST Improves Ischemic Revascularization in Aged MMP-$2^{-/-}$ Mice That Received MMP-$2^{+/+}$ BM–Derived EPCs

We observed a high blood flow signal on laser Doppler perfusion imaging in both aged MMP-$2^{+/+}$-ST and MMP-$2^{-/-}$-ST mice that received EPC-like c-Kit^+ cells derived from aged MMP-$2^{+/+}$ BM (Figure 7A). Capillary densities were also higher in the recipient mice than in the corresponding controls (Figure 7B). Cell therapy with aged MMP-$2^{-/-}$ BM abolished these ST-induced angiogenic actions in aged mice (online-only Data Supplement Figure V). To examine whether BM-derived EPC-like c-Kit^+ cells home to the ischemic vasculature, we transplanted BM from aged green fluorescent protein (GFP)–positive (GFP$^+$)-MMP-$2^{+/+}$ mice into MMP-$2^{-/-}$ mice of advanced age. Blood perfusion and numbers of c-Kit^+ cells were higher in MMP-$2^{-/-}$-ST mice than in non-ST MMP-$2^{+/+}$ mice (Figure 7C through 7E), which further suggests that a defect in MMP-2 signaling for EPC mobilization contributes in part to the decreased ischemic revascularization seen in aging without ST. Additionally, transfection GFP gene transfection had no effect on MMP-2 mRNA and activity (online-only Data Supplement Figure VI).

HIF-1α Regulates VEGF-Mediated MMP Expression in Response to Hypoxia in Human Umbilical Vein Endothelial Cells

Old human umbilical vein endothelial cells (HUVECs; 15 to 17 population doublings) had much less MMP-2 activity than young HUVECs (5 to 7 population doublings; Figure 8A), which suggests that MMP-2 transcription becomes impaired with age in vitro. To understand the mechanism by which HIF-1α stimulates MMP-2 expression via VEGF signaling in vivo, we used small interfering (si) RNAs targeting HIF-1α and VEGF in young HUVECs exposed to hypoxia. Quantitative real-time polymerase chain reaction analysis demonstrated that hypoxia stimulated MMP-2 mRNA expression; this effect was attenuated by siHIF-1α-07, siHIF-1α-09, and siVEGF, as well as their combinations (siHIF-1α-07 plus siVEGF or siHIF-1α-09 plus siVEGF; Figure 8B). In addition, hypoxia stimulated the gelatinolytic activity of MMP-2; this effect was also diminished by the siRNAs singly or in

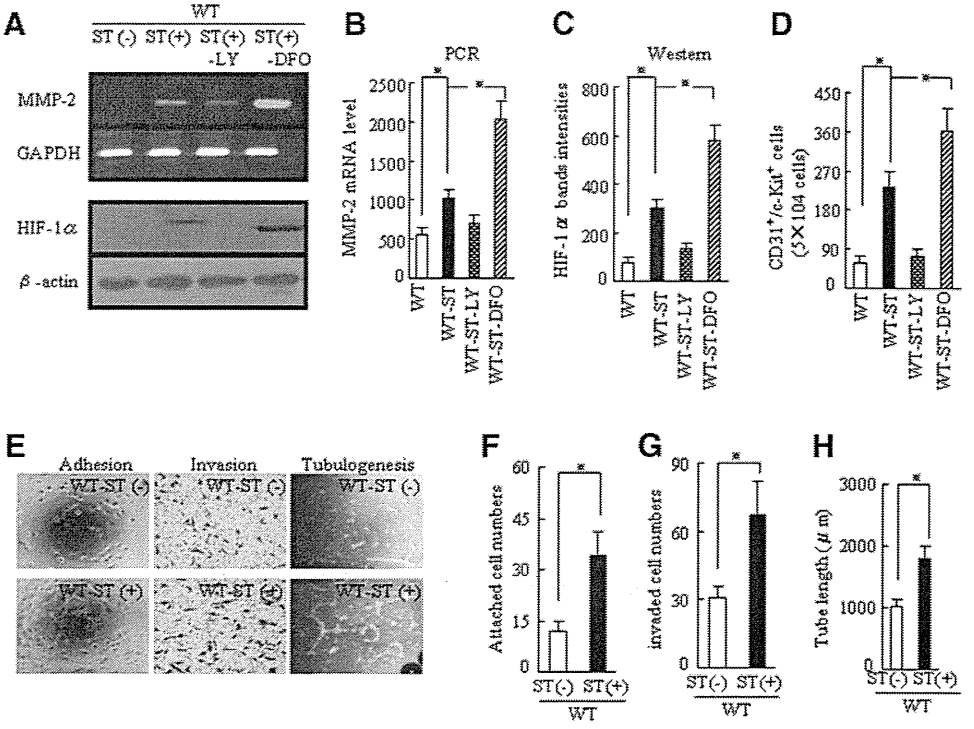

Figure 6. ST stimulates MMP-2 and HIF-1α expression in BM-derived EPCs and improves EPC function in the WT, WT-ST, WT-ST-LY, and WT-ST-DFO groups on postoperative day 8. A, Representative polymerase chain reaction (PCR) and Western blots showing expression of MMP-2 mRNA and HIF-1α protein in the 4 indicated groups. B–D, Quantitative analysis of levels of MMP-2 mRNA and HIF-1α protein and the numbers of $CD31^+$/c-Kit^+ cells in the 4 groups (*$P<0.05$, Kruskal-Wallis and Tukey post hoc tests). E, Cellular function assays showing the beneficial effects of ST on the indicated cellular events in EPCs. F–H, Quantitative analysis of the capability for adhesion, invasion, and tubulogenesis for EPCs from the 4 groups (*$P<0.05$, Student *t* test).

combination (Figure 8C and 8D). These findings suggest that HIF-1α can regulate VEGF-dependent MMP-2 expression and activity in vascular endothelial cells.

siHIF-1α Impairs HUVEC Invasion and Tubulogenesis in Response to Hypoxia

Because HIF-1α supports EPC mobilization and the angiogenic response in vivo,[17] we speculated that disruption of HIF-1α activity may influence cellular function. In hypoxic conditions (1% O_2), siHIF-1α-07 attenuated the numbers of invading HUVECs in a cell invasion assay (online-only Data Supplement Figure VIIc and VIId). Similarly, siHIF-1α-07 inhibited the HUVEC tubulogenic response (online-only Data Supplement Figure VIIe). These findings provide evidence that hypoxia-induced HIF-1α may support revascularization by affecting EPC function.

Discussion

Here, we report the novel finding that ST stimulates the PI3K/Akt-dependent HIF-1α activation pathway and attenuates the PHD/FIH-mediated HIF-1α degradation pathway in ischemic tissues and within the cells of aged animals. The increase in HIF-1α transcriptional activity increased the expression of the angiogenic genes VEGF, Flt-1, and MMP-2 in ischemic tissue and BM-derived progenitor cells, thereby improving not only recruitment of EPCs but also their function. This resulted in a substantial number of circulating $CD34^+$/c-Kit^+ EPC-like cells and their homing to the vasculature, accelerating ischemic revascularization and blood flow recovery.

The ability of ST to increase HIF-1α levels is likely to contribute to the stimulation of revascularization under experimental conditions. The ability of HIF-1α to activate the expression of multiple angiogenic growth factor genes as a direct transcriptional activator has been demonstrated previously.[20] Here, ST increased the protein levels of VEGF and Flt-1 as well as HIF-1α in ischemic muscles of aged WT mice. It has been reported previously that insulin or IGF-1 enhances HIF-1α activation via stimulation of the PI3K/Akt signaling pathway in several cancer lines.[18,21] A recent study demonstrated that the IGF-1/IGF-1R signaling pathway may serve redundant roles in the cardiac response to ST.[22] Here, ST increased IGF-1 protein levels in ischemic muscle, and the ability of ST to enhance beneficial vascular action was abrogated by LY in vivo. Thus, the increase in HIF-1α transcriptional activity by IGF-1–mediated activation of the PI3K/Akt signaling pathway could represent an ST-related mechanism that protects cardiovascular tissue from stress.

On the other hand, the present data show that HIF-1α stability is also required for ST-stimulated revascularization in ischemic tissue. The PHD family of proteins degrades HIF-1α,[23] and ST inhibited increases in the levels of PHD-3 and FIH proteins in ischemic tissues. DFO is an HIF-1α stabilizer that prevents HIF-1α degradation by several PHD families,[23,24] and DFO enhanced the ST-induced increase in ischemic muscular HIF-1α levels and angiogenic response to ischemia but slightly reduced the ST-related effects on PHD-3 and FIH. DFO has also been shown to stimulate angiogenic cytokines in aged animals.[23] These findings provide evidence that the vasculoprotective action of ST is mediated, at least in part, through the increase in HIF-1α stabilization that occurs when PHD3 and FIH expression is reduced.

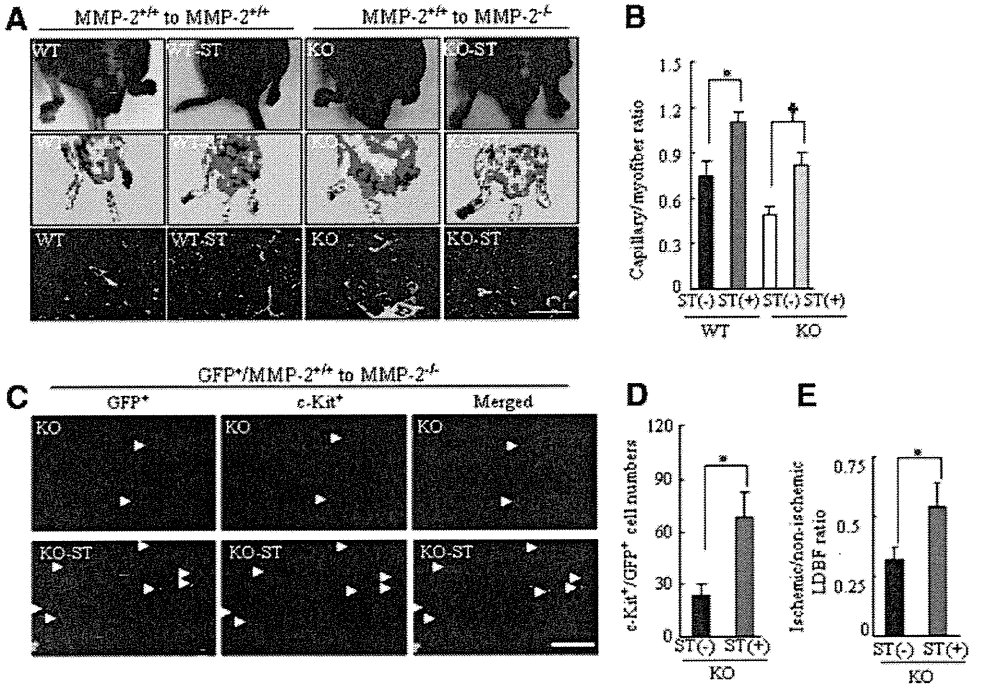

Figure 7. ST improves the effects of cell therapy on ischemic revascularization in both genotypes mice at age 24 months. A, Pictures of legs, laser Doppler imaging, and immunostaining showing ST-mediated protection against spontaneous amputation of ischemic feet and improvement of blood perfusion and capillary density in MMP-$2^{+/+}$ and MMP-$2^{-/-}$ mice that received BM cells from 18-month-old MMP-$2^{+/+}$ mice. B, ST also reversed the impairment of revascularization in MMP-$2^{+/+}$ or MMP-$2^{-/-}$ mice that received cell therapy (n=5 per group; *P<0.05, 2-way ANOVA). C, Fluorescence staining with an anti–c-Kit monoclonal antibody (red) showing EPC homing into the vasculature of ischemic tissues of MMP-$2^{-/-}$ mice that received GFP$^+$ MMP-$2^{+/+}$ BM without or with ST. D and E, Quantitative analysis of the numbers of c-Kit$^+$ EPCs and the ratio of ischemic-nonischemic laser Doppler blood flow in KO and KO-ST groups (n=5 per group; *P<0.05, Student t test).

It has become clear that the function and the number of EPCs are modified by pathological conditions such as aging and cardiovascular disease and by therapeutic exercise intervention.[5,13] In the present observations, the adhesiveness, invasiveness, and tubulogenesis responses of BM-derived EPCs to VEGF were higher in WT-ST mice than in WT mice; these effects were abrogated by LY and enhanced by DFO. Furthermore, siHIF-1α impaired HUVEC invasion and tubulogenic responses in vitro. Partial HIF-1α deficiency and impaired HIF-1α stabilization decreases EPC recruitment and revascularization in young and aged animals.[17,23] Thus, the ability of ST to improve EPC function has a salutary effect on the vasculature under conditions of ischemic stress by enhancing HIF-1α activity pathways in EPCs, thereby promoting revascularization.

The present findings are consistent with the results of Chang et al,[23] who demonstrated an impairment of BM-mediated vasculogenesis in the ischemic tissues of aged mice; however, unlike in the present study, they found no effects of age on EPC number and function in mice. This difference may be due to differences in the ischemic models or to differences in the cell type or specific cellular functions. Chang et al[23] used the skin flap model for their evaluation. Furthermore, they studied only the migration of EPCs, defined as Flk-1^+/CD11^- cells, in healthy younger and older animals. In contrast, we used a more extensive hindlimb ischemic model, and we defined CD31^+/c-Kit$^+$ cells as EPCs and studied both their invasion and tubulogenic response. This may have provided a more robust determination of differences in EPC number and function.

Among the members of the MMP family, MMP-2 is sensitive to the hypoxia caused by ischemia.[5,25] Recently, we have shown that exposure to VEGF or basic fibroblast growth factor causes the predominant accumulation of MMP-2 transcripts in vascular endothelial cells.[5,6] In the data presented here, ST stimulated MMP-2 mRNA expression in ischemic muscle and EPCs of MMP-$2^{+/+}$ mice; these changes were abolished by ST-LY and enhanced by ST-DFO. In HUVECs, aging impairs MMP-2 expression and activity, and both siHIF-1α and siVEGF reduced MMP-2 gene and protein expression. Because genetic deletion or selective pharmacological inhibition of MMP-2 inhibits angiogenesis in vitro and vivo,[5,6] we propose that the hypoxia-induced MMP-2 reactivation by stimulation of the PI3K/HIF-1α/VEGF signaling pathway may be responsible for the vasculoprotective action of ST. This notion is further supported by the lack of effect of ST on angiogenesis in MMP-$2^{-/-}$ mice. More importantly, after transplantation with c-Kit$^+$ EPC-like cells derived from aged MMP-$2^{+/+}$ BM, ST restored blood perfusion and capillary formation in aged MMP-$2^{-/-}$ mice. MMP-2 deficiency impairs the mobilization of CD31^+/c-Kit$^+$ EPC-like cells in young and old animals.[5] These data suggest that that ST-mediated improvement of vascularization may be attributable to the increase of EPC mobilization and to their functional incorporation into the vasculature in MMP-$2^{-/-}$-ST mice.

The data presented here show that aging promotes the formation of pathological vessels with irregular lumens. Previous studies have reported that the reduction of PI3K/Akt expression or inhibition of HIF-1α activity can contribute to

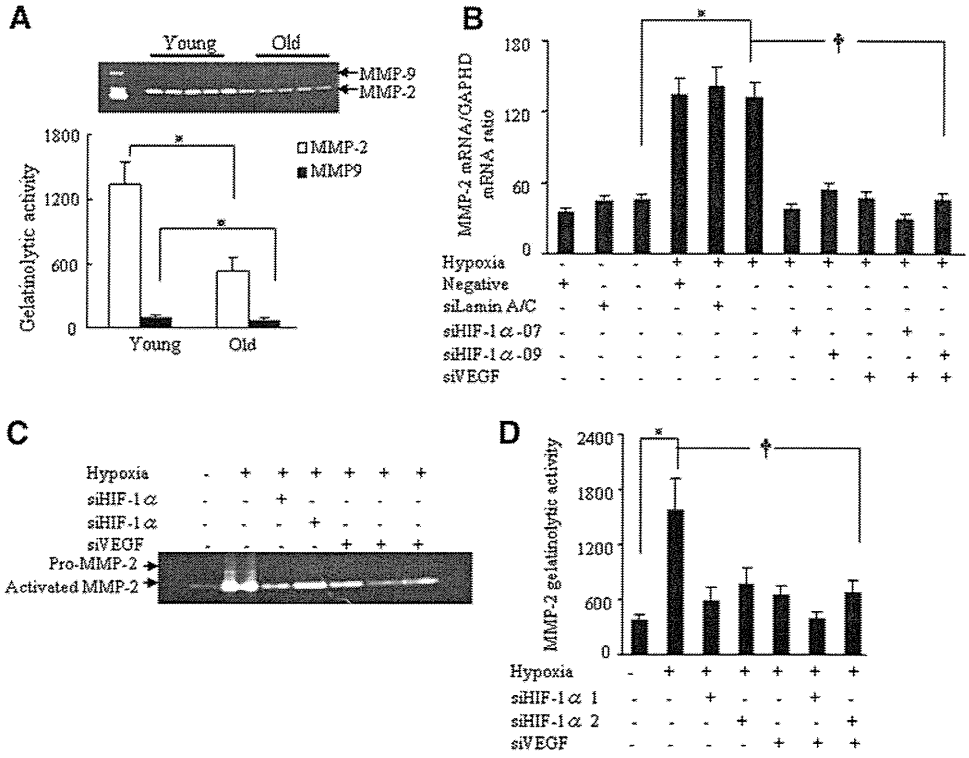

Figure 8. HIF-1α regulates VEGF-dependent MMP-2 expression in response to hypoxia in HUVECs. A, Representative gelatin zymography (upper panel) showing that VEGF induces MMP-2 and MMP-9 activities in the conditioned media of young and old HUVECs. Quantitative analysis of the zymography (lower panel) showed the gelatinolytic activities of MMP-2 and MMP-9 (*P<0.05, 3 independent experiments, Student t test). B, Quantitative analysis of MMP-2 mRNA expression in the lysates of young HUVECs treated with or without a nontargeting siRNA, siRNA-lamin A/C (siLamin A/C), siHIF-1α-07, siHIF-1α-09, or siVEGF under hypoxic conditions (*P<0.05, †P<0.05, Kruskal-Wallis and Tukey post hoc tests; 6 independent experiments). C, Representative gelatin zymography showing the inhibitory effects of targeted siRNAs on MMP-2 activity. D, Quantitative analysis of MMP-2 activity in the conditioned media of HUVECs treated with or without the indicated siRNAs (*P<0.05, †P<0.05, Kruskal-Wallis and Tukey post hoc tests; 3 independent experiments).

vessel maturation in gastric tumors.[26] In the present study, ST reduced the formation of abnormal vessels and enhanced vessel maturation in ischemic muscle, and the ability of ST to favorably affect angiogenesis was abrogated in WT-ST-LY mice and was enhanced in WT-ST-DFO mice. Conversely, ST promoted collateral artery formation in the ischemic limbs of aged WT mice; this change was abolished by ST-LY and was enhanced by ST-DFO. We hypothesize that ST prevented the aberrant vascular structures and enhanced collateral artery formation by increasing HIF-1α activity, which resulted in improvement of blood perfusion recovery in ischemic limbs of aged WT mice. To the best of our knowledge, this is the first report to have clearly demonstrated that ST significantly enhances functional therapeutic neovascularization.

The present study confirmed the dysfunctional hypoxia response in aged ischemic tissue with subsequent impairment in downstream gene expression (VEGF, Flt-1, and MMP-2), offering a potential explanation for the disappointing outcome of clinical trials with cell therapies to date. Therapeutic interventions with exercise training in advanced age designed to restore the "young" hypoxic response can be recommended as a powerful strategy to prevent age-associated declines in vascular regeneration and function by recruiting and improving delivery of EPCs to the vasculature of ischemic tissues through PI3K signaling pathway–dependent HIF-1α/VEGF/MMP-2 activation. Future studies with patients of advanced age will be required to investigate whether healthy subjects and specific patient subsets with peripheral artery disease or coronary artery disease achieve the potential vascular benefit with exercise. This might also allow the therapeutic use of nonpharmacological interventions, such as voluntary exercising, to complement pharmacological or genetic interventions such as statins or vascular-related gene therapy.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Aging is associated with a decreased ability to form new vasculature in response to hypoxia, which results in diminished capacity for tissue regeneration. Previous clinical and experimental investigations have shown that exercise stimulates the vascular response to pathological conditions in humans and animals of young or advanced age; however, the molecular mechanisms by which exercise improves the aging-associated impairment in the hypoxia-induced factor (HIF)-1α–mediated response to hypoxia are poorly understood. In the present study, we first showed that swimming training (ST) modulates the vascular response to ischemia in aged mice. ST improved the age-impaired recovery of not only blood reperfusion and capillary formation but also the levels of p-Akt, HIF-1α, vascular endothelial growth factor, and matrix metalloproteinase-2 in wild-type mice of advanced age. ST also improved collateral vessel formation and reduced pathological vessel formation and foot amputation. Furthermore, data from cell therapy experiments confirmed that ST stimulates the mobilization of endothelial progenitor cells and the homing of these cells to the site of the ischemic vasculature associated with activation of HIF-1α. All of these effects were diminished by LY2940029, an inhibitor of phosphatidylinositol 3-kinase; enhanced by deferoxamine, an HIF-1α stabilizer; and impaired by knockout of matrix metalloproteinase-2. Thus, therapeutic interventions with ST in advanced age restore the "young" hypoxic response and prevent age-associated declines in vascular regeneration by recruiting and improving delivery of endothelial progenitor cells to the vasculature through phosphatidylinositol 3-kinase signaling pathway–dependent HIF-1α/vascular endothelial growth factor/matrix metalloproteinase-2 activation. We therefore conclude that a therapeutic ST intervention represents the complement of pharmacological or genetic interventions.

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Matrix metalloproteinase-2 regulates the expression of tissue inhibitor of matrix metalloproteinase-2

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SUMMARY

1. Matrix metalloproteinases (MMP) are associated with the vascular remodelling seen in atherosclerosis and aneurysm. The activation and activity of MMP-2 are regulated by the intrinsic tissue inhibitor of MMP-2 (TIMP-2). The aim of the present study was to examine whether, conversely, MMP-2 can affect the gene and protein expression of TIMP-2.

2. In the present study, we examined the mRNA and protein expression of MMP-2 and TIMP-2 in cultured smooth muscle cells (SMC) from the aortas of MMP-$2^{+/+}$ and MMP-$2^{-/-}$ mice. We also examined the roles of MMP-2 in SMC cellular events.

3. Western blotting showed that less TIMP-2 protein was present in the conditioned medium of MMP-$2^{-/-}$ SMC than in that of MMP-$2^{+/+}$ SMC. Real-time reverse transcription polymerase chain reaction analysis showed that MMP-2 deficiency reduced TIMP-2 mRNA expression in SMC. Recombinant MMP-2 enhanced the expression of TIMP-2 protein in cultured SMC from MMP-$2^{-/-}$ mice. Furthermore, a siRNA targeting MMP-2 impaired the gene and protein expression of MMP-2 in cultured SMC from MMP-$2^{+/+}$ mice. MMP-2 deficiency impaired SMC invasion, but not their proliferation, adhesion or migration.

4. Our findings suggest that MMP-2 is likely to be responsible, at least in part, for regulating TIMP-2 expression and is thus a potential target, in addition to TIMP-2, for therapeutics aimed at preventing cardiovascular remodelling in response to injury.

Key words: matrix metalloproteinase-2, smooth muscle cell, tissue inhibitor of matrix metalloproteinase-2.

INTRODUCTION

Because vascular smooth muscle cells (SMC) in the large vessels are usually surrounded by and embedded in extracellular matrix (ECM) proteins, the migration of SMC and the remodelling of tissues during atherogenesis require controlled degradation of the ECM.[1–3] SMC can produce proteolytic enzymes, such as matrix metalloproteinases (MMP), a family of zinc- and calcium-dependent proteinases that degrade collagen and other matrix proteins. MMP are involved in the accelerated breakdown of the ECM associated with vascular remodelling during the pathogenesis of atherosclerosis and aneurysm.[4–7] Various kinds of MMP are upregulated and activated in atherosclerotic lesions, as well as in ischemic tissues in humans and animal models.[8–13] Recent efforts have focused on finding ways to control the activation and activity of MMP through tissue inhibitors of MMP (TIMP).[14] There is accumulating evidence that changes in the MMP/TIMP balance might play an important role in the structural, functional and clinical manifestations of hypertensive cardiovascular disease.[15–17] An important yet elusive goal has been to determine which part of this balance is critical in cardiovascular remodelling and neovascularization in response to injury. However, *in vivo* experiments have provided little insight into how to control the MMP/TIMP balance.

Among MMP, MMP-2 (also called gelatinase A) is a major MMP derived from vascular cells that degrades various ECM proteins and such barriers as the basement membrane.[1,2] MMP-2 and TIMP-2 are upregulated in human atherosclerotic lesions, as well as in various animal models of neointimal formation.[11,17,18] We previously showed in MMP-$2^{-/-}$ mice that MMP-2 deficiency significantly reduces the levels of TIMP-2 mRNA and protein in atherogenic lesions of carotid arteries or ischemic tissues of the muscle relative to the levels in MMP-$2^{+/+}$ mice.[8] Although it has been reported that MMP-2 activation and activity are mainly controlled by TIMP-2 in vascular cells *in vivo* and *ex vivo*, [19–21] there is no direct evidence that MMP-2 regulates TIMP-2 expression. To our knowledge, the present study investigates for the first time whether MMP-2 regulates the expression of the TIMP-2 gene and protein in cultured vascular SMC.

METHODS

Cell isolation and culture

SMC were isolated from the aortas of wild-type (MMP-$2^{+/+}$, C57/BL6) and MMP-2 knockout (MMP-$2^{-/-}$, C57/BL6 background) mice by the explants method,[8] and were cultured with Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Mouse aortic SMC were subcultured at passages 4–11 for the following experiments, as previously described.[8]

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The SMC were plated in six-well plates at 2.0×10^5 cells/well in 2 mL of 10% FBS/DMEM (v/v) and incubated overnight. The cells were then incubated with serum-free DMEM for 24 h, after which the conditioned media and the cellular extracts were subjected to gelatin zymography, western blotting and real-time reverse transcription polymerase chain reaction (RT–PCR) analysis. To evaluate the effect of MMP-2 protein on TIMP-2 expression, SMC were treated with 0.5 μg/mL mouse recombinant MMP-2 (rMMP-2; Oncogene Research Products, Cambridge, MA, USA) in serum-free DMEM for 24 h, and the conditioned medium was collected for western blot analysis.

Gene expression assay

Total RNA was extracted from cell extracts using a RNeasy Mini kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. mRNA was reverse transcribed to cDNA with the RNA PCR Core kit (Applied Biosystems, Foster City, CA, USA). Quantitative gene expression was studied with the ABI 7300 Real-Time PCR System (Applied Biosystems) with TaqMan Universal PCR Master Mix (Applied Biosystems). All experiments were carried out in triplicate. Each RNA quantity was normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA quantity measured in the same RNA preparation. The sequences of primers and probes for the targeted mouse MMP (MMP-2, MMP-3, MMP-9, MMP-14) and TIMP (TIMP-1, TIMP-2) were described previously.[8]

Gelatin zymography

For gelatin zymography, 20-μg protein extracts of SMC-conditioned media were mixed with sodium dodecyl sulfate (SDS) sample buffer without reducing agent and loaded onto a 10% SDS-polyacrylamide gel containing 1 mg/mL gelatin as a substrate, as previously described.[22] In brief, after electrophoresis, the gels were washed twice with 2.5% Triton X-100 and incubated in buffer containing 50 mmol/L Tris (pH 8.0), 50 mmol/L NaCl and 10 mmol/L $CaCl_2$ overnight at 37°C. After the gel was stained with Coomassie Brilliant Blue, the digestion bands were quantified with an image analyser system (NIH Image 1.62, Bethesda, MD, USA). Recombinant human activated MMP-2 (Oncogene Research Products) was used as a positive control.

Western blot analysis

The protein concentration for each sample was determined using a protein assay system (Bio-Rad DC; Bio-Rad Laboratories, Hercules, CA, USA) in accordance with the manufacturer's instructions. Equal amounts of total protein (80 μg/lane) were separated in a 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. After reaction with primary antibodies against MMP-2 (Fuji Chemical, Toyama, Japan), MMP-9 (Chemicon, International, Temecula, CA, USA), and TIMP-1 and TIMP-2 (both from Sigma-Aldrich, St. Louis, MO, USA), the membranes were treated with peroxidase-linked anti-mouse IgG or peroxidase-linked protein A (Amersham Biosciences, Buckinghamshire, UK). Band intensity was quantified with an image analyser system (NIH Image 1.62) as previously described.[23]

Transfection of small interfering RNA

Cells were electroporated by the Amaxa nucleofection method (Amaxa, Cologne, Germany) with Nucleofector reagent for SMC. siRNA (2 μmol/L) was introduced into 1×10^6 cells through nucleofection using Basic Nucleofector Solution (Amaxa Ucleofector kit, Lonza, Switzerland) and the D-33 program. After nucleofection, the cells were replated in six-well plates at 3.0×10^5 cells/well in 2 mL of 10% FBS/DMEM. After overnight incubation, the media were replaced with serum-free DMEM and incubated for 48 h. At the end of the incubation, the conditioned media were collected and concentrated for gelatin zymography and western blot analysis, and total cellular RNA was extracted for real-time RT–PCR analysis. Additionally, after treatment with siMMP-2 for 24 h, MMP-$2^{+/+}$ SMC were cultured in the presence or absence of rMMP-2 for 24 h and subjected to real-time PCR for TIMP-2 expression. Two siRNA (NIPPON EGT, Toyama, Japan) were used for the present study: siRNA-1 (a, 494), 5′-ACCUCUUUGUGCUGAAAGAdTdT-3′ (sense) and siRNA-2 (b, 1465), 5′-UGUCCUGACCAAGGAUAUAdTdT-3′ (sense), 5′-UAUAUCCUUGGUCAGGACAdTdT-3′ (anti-sense). Non-siRNA was used as a negative control. In addition, maxGFP was used as a positive control to examine the effect of green fluorescent protein gene transfection on MMP-2 mRNA expression in SMC.

Migration and invasion assays

The migration assay was carried out with Transwell (Costar) 24-well tissue-culture plates composed of a polycarbonate membrane containing 5-μm pores, as previously described.[24] The membrane was coated with 50 μg/mL collagen type I solution in 0.02 N acetic acid for 12 h at 4°C and then rinsed well with phosphate buffer saline. SMC were seeded on the inner chamber of the Transwell at 106 cells in 100 μL of DMEM containing 0.3% bovine serum albumin (BSA). The inner chamber was placed into the outer chamber, which contained 600 μL of DMEM containing 0.3% BSA supplemented with recombinant human platelet-derived growth factor-BB (PDGF-BB, 10 ng/mL), and then incubated for the indicated periods of time at 37°C in a CO_2 incubator. Cells that migrated onto the outer side of the membrane were fixed and stained with Dif-Quick stain (International Reagents Corp, Kobe, Japan). The number of cells that had migrated was counted in six to eight randomly chosen fields of duplicate chambers at × 200 magnification for each sample.

The invasion assay was carried out in a similar manner, but with a coating of fibrillar collagen. In brief, 1.0 mg/mL collagen type I solution in 0.02 N acetic acid and a 1/10 volume of 10 × DMEM were mixed and neutralized with 1 N NaOH at 4°C. A total of 20 μL of the mixture was added to an inner-chamber membrane and polymerized at 37°C for 6 h. The SMC suspension in DMEM containing 0.3% BSA was then added to the inner chamber as described earlier.

SMC proliferation assay

SMC proliferation was assessed with a Cell Titer 96AQ Assay kit in accordance with the instructions of the manufacturer (Promega, Tokyo, Japan). SMC were plated on collagen-coated 96-well plates at 5000 cells in 100 μL of DMEM with 0.3% BSA per well. After 2 h of incubation, the medium was replaced with DMEM in the presence or absence of 20% FBS or PDGF (20 ng/mL), followed by another 3 days in culture. Then 20 μL of a mixture of tetrazolium compound and phenazine methosulfate was added, and the absorbance was determined at 492 nm as previously described.[25]

Adhesion assay

The 96-well plates were coated with matrix components as previously described. A SMC suspension in serum-free DMEM containing 0.3% BSA was plated at 2×10^4 cells per well. After 2 h of incubation, the attached cells were counted in six randomly chosen fields of duplicate wells.[3]

Statistical analysis

Data are expressed as means ± SD. Statistical analysis was carried out with the unpaired Student's *t*-test or analysis of variance followed by Scheffe's multiple-comparison post hoc test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

MMP-2 deficiency impairs the expression of TIMP-2 mRNA and protein

As expected, we did not detect MMP-2 mRNA expression in the SMC of MMP-$2^{-/-}$ mice with real-time PCR (Fig. 1a). Expression

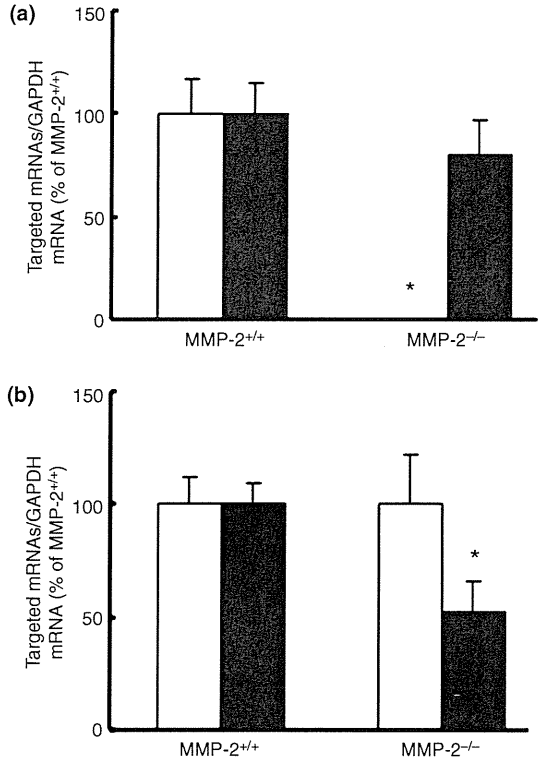

Fig. 1 Expression of matrix metalloproteinases (MMP; MMP-2 (□), MMP-9 (■)) and tissue inhibitors of MMP (TIMP; TIMP-1 (□), TIMP-2 (■)) in cultured smooth muscle cells (SMC) from the aortas of MMP-$2^{+/+}$ and MMP-$2^{-/-}$ mice. Total cellular RNA was analysed by quantitative real-time reverse transcription polymerase chain reaction for MMP-2 and MMP-9 (a; $n = 4$ per group), and TIMP-1 and TIMP-2 (c; $n = 7$ per group). Data are expressed as a percentage of mRNA levels in MMP-$2^{+/+}$ mice. *$P < 0.001$ versus MMP-$2^{+/+}$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

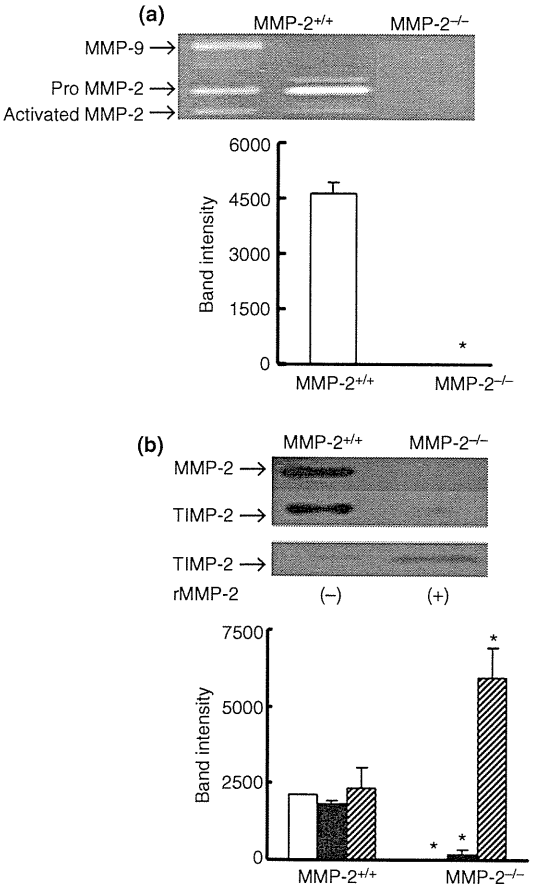

Fig. 2 Expression of matrix metalloproteinase-2 (MMP-2) and tissue inhibitors of MMP-2 (TIMP-2) proteins in the conditioned media of MMP-$2^{+/+}$ smooth muscle cells (SMC). Samples were analysed by (a) gelatin zymography for MMP-2 activity (□) and TIMP-2 (■) proteins (b, $n = 4$ per group). Mouse recombinant MMP-2 (rMMP-2) increased TIMP-2 (▨) protein expression in cultured SMC from MMP-$2^{-/-}$ mice (b; $n = 7$ per group). The values were obtained by densitometric evaluation of the gelatin zymography and western blots. Data are expressed as mean ± SD. *$P < 0.001$ versus MMP-$2^{+/+}$.

of TIMP-2 mRNA was significantly lower in SMC of MMP-$2^{-/-}$ mice than of MMP-$2^{+/+}$ mice (Fig. 1b). However, MMP-2 deficiency had no effect on the expression of MMP-9 or TIMP-1 mRNA (Fig. 1a,b). Similarly, MMP-2 deficiency had no effect on other MMP family members, such as MMP-3 and MMP-14 (data not shown).

A previous study showed that the primary MMP derived from SMC in an invasion assay is MMP-2, not MMP-9.[1] As in that previous work, in the present study we detected both the pro- and active forms of MMP-2 (72 and 62 kDa), but not MMP-9, in the SMC-conditioned media of MMP-$2^{+/+}$ mice assessed with gelatin zymography (Fig. 2a). No MMP-2 protein or gelatinolytic activity was detected in the SMC-conditioned media of MMP-$2^{-/-}$ mice (Fig. 2a,b). Similar to the effect on gene expression, the level of TIMP-2 protein was dramatically decreased in the conditioned medium of SMC from MMP-$2^{-/-}$ mice (Fig. 2b). Treatment with mouse rMMP-2 significantly enhanced the production of TIMP-2 protein in the SMC-conditioned medium of MMP-$2^{-/-}$ mice compared with the control (Fig. 2b).

siRNA-MMP-2 impairs the expressions of TIMP-2 mRNA and protein

To investigate whether MMP-2 regulates TIMP-2 expression, SMC from the aortas of MMP-$2^{+/+}$ mice were transfected with siRNA-MMP-2 and subjected to real-time PCR and gelatin zymography. Quantitative PCR showed that siRNA-MMP-2 significantly reduced the levels of MMP-2 and TIMP-2 mRNA. SiRNA-1 and -2 reduced the levels of MMP-2 by 92% and 79% and the levels of TIMP-2 by 32% and 25%, respectively, compared with the values for the corresponding controls (Fig. 3a,b). siRNA-MMP-2 had no effect on the expression of the MMP-9 and TIMP-1 genes (Fig. 3a,b).

As expected, the gelatin zymography and western blot analyses showed that siRNA-MMP-2 reduced the level of MMP-2 protein and its net gelatinolytic activity in the MMP-$2^{+/+}$ SMC-conditioned medium (Fig. 4a,b). As it had with gene expression, the siRNA-MMP-2 also impaired TIMP-2 protein production (Fig. 4b). No MMP-9 activity and TIMP-1 protein were detected in the conditioned medium of MMP-$2^{+/+}$ SMC treated with siRNA-MMP-2 (Fig. 4a, data not shown). In addition, we observed that after treatment with siMMP-2 (494), rMMP-2 recovered TIMP-2 mRNA expression in cultured MMP-$2^{+/+}$ SMC (1.56-fold over non-treated control, $P < 0.05$, data not shown).

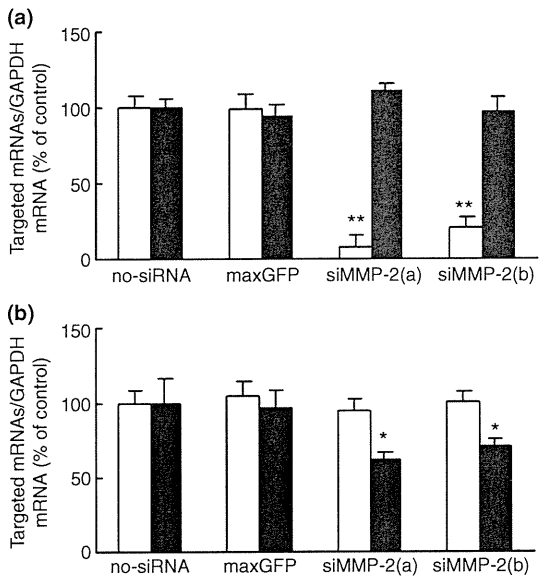

Fig. 3 Effect of siRNA-matrix metalloproteinase-2 (MMP-2) on gene expression of MMP and tissue inhibitors of MMP (TIMP) in cultured MMP-$2^{+/+}$ smooth muscle cells. Total cellular RNA was analysed by quantitative real-time reverse transcription polymerase chain reaction for (a) MMP-2 (□) and MMP-9 (■), and (b) TIMP-1 (□) and TIMP-2 (■). Data are expressed as a percentage of the corresponding control mRNA levels and are mean ± SD (n = 4 per group). *P < 0.05, **P < 0.001 versus the corresponding controls. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

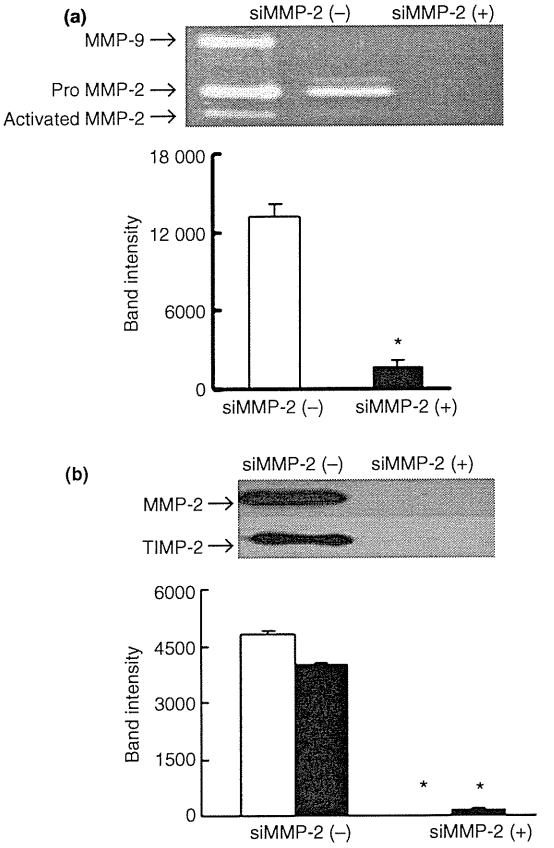

Fig. 4 Effect of siRNA-matrix metalloproteinase-2 (MMP-2) on the production of MMP-2 and tissue inhibitors of MMP-2 (TIMP-2) proteins and/or activity in the conditioned media of MMP-$2^{+/+}$ smooth muscle cells. Samples were analysed by gelatin zymographic analysis for (a) MMP-2 activity and (b) western blot analysis for the levels of MMP-2 (□) and TIMP-2 (■) proteins. The values were obtained by densitometric evaluation of the gelatin zymography and western blots. Data are expressed as mean ± SD (n = 4 per group). *P < 0.001 versus the corresponding controls.

Effects of MMP-2 deficiency on SMC cellular functions

To test whether MMP-2 contributes to SMC proliferation, SMC isolated from MMP-$2^{+/+}$ and MMP-$2^{-/-}$ mice were used in a proliferation assay. MMP-2 deficiency had no effect on the proliferation of SMC stimulated with serum-free DMEM, 2% FBS or 20 ng/mL PDGF (Fig. 5a). There was no difference in adhesive action between SMC from MMP-$2^{+/+}$ and MMP-$2^{-/-}$ mice (Fig. 5b,c). Furthermore, MMP-2 deficiency also produced no effect on SMC migration across a filter lightly coated with type I collagen (Fig. 5b,c). However, MMP-2 deficiency impaired SMC invasion through a thick layer of collagen lattice (Fig. 5b,c).

DISCUSSION

As their name implies, TIMP are endogenous inhibitors of MMP activity and as such were initially thought to function principally to modulate MMP activity and suppress ECM turnover. However, accumulating evidence shows that TIMP have MMP-independent biological activities. A previous study reported that TIMP-1 expression is inversely correlated with the susceptibility of various human Burkitt's lymphoma cell lines to the induction of programmed cell death (apoptosis).[26] Recombinant TIMP-1 or forced expression of TIMP-1 reduces the induction of apoptosis, suppresses caspase-3 activity and sustains DNA synthesis under serum-free conditions in a TIMP-negative cell line.[26] TIMP-1 and TIMP-4 inhibit apoptosis and anoikis in human breast epithelial cells *in vitro*.[27,28] Furthermore, several distinct signalling pathways have been implicated in the growth-promoting activity of TIMP, including the mitogen-activated protein kinase (MAPK) and adenosine 3′,5′-monophosphate (cAMP)-protein kinase A pathways.[29,30] A recent report shows that TIMP-1 induces cell cycle arrest in G_1 in association with the down-regulation of cyclin D_1, upregulation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ and hypophosphorylation of the retinoblastoma protein.[31] The growth-promoting activities of TIMP-1 and TIMP-2 require the activation of Ras, albeit by distinct pathways, suggesting independent receptor mechanisms.[32] In contrast, TIMP-2 binding to the endothelial cell surface and its ability to inhibit endothelial cell proliferation are independent of MMP inhibition, as shown by Ala+TIMP-2.[33] Subsequent studies showed that TIMP-2 or Ala+TMIP-2 binding to the αvβ3 integrin as a receptor results in G_1 growth arrest and enhanced de novo expression of $p27^{Kip1}$.[34] These findings suggest that TIMP are multifunctional and can act to direct cell fate either directly through cell surface receptors or indirectly through modulation of protease activity. The emerging concept is that TIMP function in a contextual fashion, such that the mechanism of action depends on the tissue microenvironment.

We have shown that MMP-2 deficiency reduces the levels of TIMP-2 mRNA and protein in atherogenic lesions of carotid arteries and ischemic muscle tissues in MMP-$2^{-/-}$ mice.[4,8,13] MMP-2 deficiency and a siRNA targeting MMP-2 also reduced the levels of TIMP-2 mRNA and protein in cultured SMC. Furthermore, rMMP-2 enhanced TIMP-2 protein production in MMP-$2^{-/-}$ SMC.

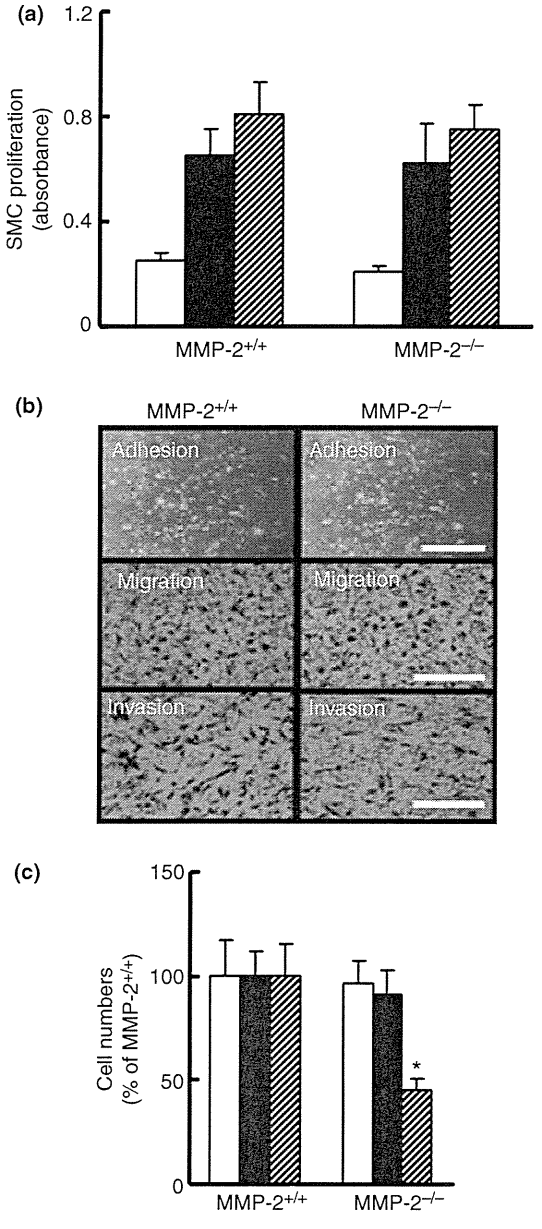

Fig. 5 Effect of matrix metalloproteinase-2 (MMP-2) on smooth muscle cells (SMC) proliferation, adhesion, migration and invasion. (a) MMP-2 deficiency had no effect on SMC proliferation in the presence of Dulbecco's modified Eagle's medium (DMEM (□)), 2% fetal bovine serum (■) or 20 ng/mL platelet-derived growth factor (▨). (b,c) MMP-2 deficiency reduced SMC invasion (▨), but not adhesion (□) or migration (■). Data are expressed as mean ± SD (n = 4 mice per group). *P < 0.001 versus the corresponding controls. Bar, 100 μm.

However, the genetic interventions (MMP-2 gene knockout and siRNA-MMP-2 transfection) did not affect the levels of MMP-9 and TIMP-1 mRNA and proteins in cultured SMC. On the basis of these findings, we propose that a MMP-2 deficiency-mediated decrease in TIMP-2 expression might be one mechanism causing a reduction of atherogenic lesion formation and angiogenic actions in animal models.

Surface localization of the activated MMP might be crucial for cellular events, including cell adhesion, migration/invasion and proliferation, that have been associated with angiogenesis-dependent tumor morphogenesis.[35,36] Recently, studies by the present authors and others using MMP-2 knockout animals provided evidence that neointimal formation and angiogenesis are specifically mediated by MMP-2.[4,8,13] In line with these findings, *ex vivo* aortic-ring culture assays showed that MMP-2 deficiency profoundly impairs the vascular endothelial growth factor-mediated tubulogenic response in aorta explants.[13] Furthermore, MMP-2 deficiency as well as an endogenous inhibitor of MMP-2, TIMP-2, inhibited endothelial cell invasion but not its proliferation, adhesion or migration.[13] Consistent with this, in the present study, genetic deletion of MMP-2 markedly abolished the ability of SMC to invade through type I collagen gel in an SMC invasion assay. However, MMP-2 deficiency did not affect SMC adhesion, migration or proliferation. These findings suggest that MMP-2-mediated neointimal formation is not attributable to impairment of SMC adhesion, proliferation or motility, but rather to the impairment of SMC invasion. This notion is further supported by previous studies by the present authors and others that MMP-2, as well as other proteases, such as cysteine proteases, are not involved in skeletal muscle differentiation or endothelial cell proliferation.[25,37] A few studies have reported that TIMP-2 promotes cell growth independently of MMP inhibition.[30] Further studies will be required to understand the exact mechanism underlying the effect of MMP-2/TIMP-2 on vascular cell proliferation.

The present findings clearly show that genetic or biotechnical interference of the MMP-2 gene in vascular SMC results in reducing gene and protein expression of TIMP-2. The present findings suggest that MMP-2 is likely to be responsible, at least in part, for regulating TIMP-2 expression and is thus a potential target, in addition to TIMP-2, for therapeutics aimed at preventing cardiovascular remodelling in response to injury.

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Depressive symptoms of informal caregivers are associated with those of community-dwelling dependent care recipients

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ABSTRACT

Background: The relationship between care recipients' depressive symptoms and those of caregivers remains unknown. We evaluated the association between the depressive status of caregivers and that of community-dwelling disabled care recipients.

Methods: A prospective cohort study of 893 care recipients and paired caregivers was conducted. The care recipients were all eligible for a universal-coverage long-term care insurance program and their ages ranged from 65 to 104 years. They and their paired caregivers (age range 31–90 years) completed the 15-item Geriatric Depression Scale (GDS-15, score range: 0–15) assessment at baseline. The GDS-15 was used to measure the depression of caregivers and recipients with a threshold of <6/6+. The data included each care recipient's demographic characteristics, overall health status, basic activities of daily living, and comorbidities. The data also included the caregiver's demographic characteristics, including the caregiver's relationship to the recipient, and the caregiver's subjective burden as assessed by the Japanese version of the Zarit Burden Interview (ZBI).

Results: The mean GDS-15 scores of care recipients and caregivers were 6.7 points and 5.6 points, respectively. There was a positive correlation between the GDS-15 scores of caregivers and care recipients ($r = 0.307$, $p<0.001$). Multivariate logistic regression analysis adjusting for potential confounders including ZBI score indicated that the depressive symptoms of caregivers were associated with those whose care recipients were in the groups with moderate and high GDS-15 scores (OR: 1.97, 95% CI: 1.39–2.81, OR: 3.13, 95% CI: 1.87–5.24, respectively).

Conclusion: Caregivers' depressive symptoms are associated with the depressive mood of the care recipients even after adjusting for confounders including caregiver burden.

Key words: depressive mood, caregiver burden, dependent frail older people

Introduction

The current trend toward a community-based health care system means that when older people require care, much of it will be provided at home. This trend will lead to family members providing care for ill or disabled older relatives. The majority of family caregivers are older spouses and middle-aged adult children who care for a spouse or a parent with functional limitations.

Longitudinal studies have demonstrated that being a caregiver who is experiencing mental or emotional strain is an independent risk factor for psychiatric morbidity in the form of increased depression, contributes to the risk of health problems, and is an independent risk factor for mortality (Kiecolt-Glaser *et al.*, 1995; Gallicchio *et al.*, 2002). Caregiver depressive symptoms indicate a mood disturbance that can result from the stress of providing care. A number of studies were conducted to identify the factors related to the depressive status of caregivers, and they found those factors to include the care recipient's dependency with regard to activities of daily living (ADL), dementia, and behavioral disturbance and the caregiver's the relationship to the patient (Farran *et al.*, 1997; Clyburn *et al.*, 2000; Covinsky *et al.*, 2003; McCusker *et al.*, 2007; Schulz *et al.*, 2008). Although it has been reported that caring depressed recipients is associated with poor mental health of caregivers including increased

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dissatisfaction and burden (Sewitch *et al.*, 2004; Soldato *et al.*, 2008), the relationship between care recipients' depressive symptoms and those of caregivers remains largely unknown. In the present study, we evaluated the association between depression in community-dwelling frail elderly and depression in their caregivers.

Methods

Cohort participants

In the present study we employed baseline data for the care recipient and caregiver pairs in the Nagoya Longitudinal Study for Frail Elderly (NLS-FE). Japan introduced a universal-coverage long-term care insurance (LTCI) program in 2000. Under the LTCI program, each applicant's care levels are determined according to eligibility criteria. Eligibility status is classified into six levels ("needs support" and care levels 1–5) via the estimation of care needs based on an assessment of the current physical and mental status of the patient and his or her use of medical procedures (Campbell and Ikegami, 2000; Tsutsui and Muramatsu, 2005).

The NLS-FE was designed to compare the outcomes of different uses of the community-based care services provided by the LTCI program (Kuzuya *et al.*, 2006a; 2006b). The study population consisted of 1875 community-dwelling frail elderly (632 men and 1243 women, age 65 years or older) with some degree of physical or mental disability who were eligible for the LTCI program and lived in Nagoya City, Japan. They received various kinds of community-based services from the Nagoya City Health Care Service Foundation for Older People, which has 17 visiting nursing stations associated with care-managing centers. These 1875 NLS-FE participants and 1502 caregivers (owing to the lack of a primary caregiver for 373 of the 1875 participants) were enrolled between 1 December 2003 and 31 January 2004. They were scheduled to undergo comprehensive in-home assessments by trained nurses at baseline and at 6, 12 and 24 months. At 3-month intervals, data were collected about any important events in the lives of the participants, including mortality, admission to hospital for acute illness, or institutionalization in long-term care facilities during the 3-year follow-up. Written informed consent for participation was obtained from the participants, care recipients and caregivers, or, for those with substantial cognitive impairment, from a surrogate (usually the closest relative or legal guardian) according to procedures approved by the institutional review board of Nagoya University Graduate School of Medicine.

Data collection

The data were collected at the clients' homes through standardized interviews with care recipients or their surrogates and caregivers, and from care-managing center records taken by trained nurses. The data included each participant's demographic characteristics, general socioeconomic status, living arrangements, use of medical services, and overall health or nutritional status. When the participants were unable to answer or had cognitive impairment, surrogates and caregivers were asked. The data also included depressive symptoms as assessed by the 15-item Geriatric Depression Scale (GDS-15) (range: 0–15, with higher values indicating more depressive symptoms) (Yesavage, 1997), the presence of behavioral disturbance in the care recipient according to the primary assessment dataset of the public LTCI, and a rating for ten basic activities of daily living (bADL) (feeding, mobility in bed, bathing, grooming, dressing, using the toilet, walking inside and outside, transferring, and using stairs) using summary scores ranging from 0 (total disability) to 20 (no disability). Information on the following physician-diagnosed chronic conditions was obtained from care-managing center records: cerebrovascular disease, dementia, hypertension, neurodegenerative disorders, and other diseases comprising the Charlson Comorbidity Index (Charlson *et al.*, 1987), which represents a sum of weighted indexes and takes into account the number and seriousness of pre-existing comorbid conditions (range: 0–19, with a higher value indicating higher comorbidity).

Data were also obtained from caregivers concerning their own personal demographic characteristics including the caregiver's relationship to the care recipient (spouse, adult child, daughter-in-law or other), depressive symptoms as assessed by the GDS-15, and the caregiver's subjective burden as assessed by the Japanese version of the Zarit Burden Interview (ZBI) (Arai *et al.*, 1997), which is a 22-item self-reported inventory that examines the burden associated with functional behavioral impairments in the home care situation (range: 0–88, with higher values indicating a greater burden).

Subjects for analysis

The study population consisted of 893 community-dwelling disabled elderly (337 men and 556 women, age range: 65–104 years) and paired caregivers (213 men, 680 women, age range: 31–90), made up of those who completed the GDS-15 assessment at baseline among the 1502 pairs. Of these 1502 care recipients, 389 could not complete the GDS-15 because of severe cognitive impairment or communication impairment. Among

the 1502 caregivers, 330 could not complete or refused to take part in the GDS-15. Compared with participants, 389 care recipients who could not complete the assessment had lower mean bADL scores (mean ± SD, 10.6 ± 7.5 vs 12.9 ± 5.9, $p < 0.001$), higher prevalence rate of behavioral problems (24.5% vs 15.1%, $p < 0.001$) and dementia (51.0% vs 30.5%, $p < 0.001$), Compared with participants, 330 caregivers who were excluded in the analysis were younger (mean ± SD, 63.1±12.4 vs 64.8 ± 12.6, $p = 0.003$) and had higher score o ZBI scores (mean ± SD, 31.3 ± 17.2 vs 28.0 ± 17.0, $p = 0.002$).

Statistical analysis

To evaluate the relationship between the GDS-15 scores of caregivers and those of care recipients, Spearman's rank correlation coefficient was used. Partial rank correlation coefficients adjusted for the age and gender of the care recipients and caregivers, the caregiver relationship to the care recipient (spouse or nonspouse), bADL scores and chronic diseases of the care recipients, and ZBI scores of the caregivers were also used to measure the relationships between the GDS-15 scores of the caregivers and care recipients. Student's t-test was used to determine differences in the GDS-15 scores of caregivers between those whose care recipients had chronic disease and those whose care recipients did not.

GDS-15 scores were categorized into three groups: 0–5 points (lowest), 6–10 points (modest), and ≥11 points (highest group). Comparing the Japanese version of the GDS-15 with a psychodiagnostic interview, a cut-off score of 6 yields the highest sensitivity and specificity (Wada *et al.*, 2004). Therefore, this cut-off was used in the present study. Univariate and multivariate logistic regression models were used to assess the independent predictors of the caregivers' depressive moods, defined as a GDS-15 score of 6 or higher. The following baseline data were used in univariate analysis: (i) the care recipients' data including gender, age, GDS-15 score, bADL score, presence or absence of behavioral problems and neurodegenerative disorders, and Charlson comorbidity index; (ii) the caregivers' data including gender, age, relation to care recipient (spouse or nonspouse (models 1 and 2); daughter-in-low, spouse, adult child or other (model 3)), and ZBI score. The covariates included in the multivariate analysis were variables associated with dependent variables where $p < 0.1$ in univariate analysis. The risk of a variable was expressed as an odds ratio (OR) with a corresponding 95% confidence interval (CI).

All analyses were performed using the Statistical Package for the Social Sciences (SPSS) Version 16.0. A probability value of 0.05 or less was considered significant.

Results

Characteristics of care recipients and caregivers

Table 1 shows the characteristics of the care recipients and caregivers. The mean age of the 893 care recipients was 80.4 years, with 31.4% of the total aged 85 years or older, and 62.3% were women. The mean GDS-15 score of the care recipients was 6.7 points, while 59.6% of the total had a score of 6 or higher, and 15.7% of the total had a score of 11 points or higher. Among the care recipients, 15.1% had behavioral problems, 36.9% had cerebrovascular disease and 30.5% showed dementia. The mean age of the caregivers was 64.8 years, and approximately 27% of the caregivers were 75 years or older. The caregivers were predominantly women and family members (45.4% were spouses). The mean GDS-15 score of the caregivers was 5.6 points, while 46.5% of the total had a score of 6 or higher and 12.5% had a score of 11 or higher.

As shown in Table 2, there were positive correlations between the bADL scores or ZBI scores of the caregivers, and both the GDS-15 scores of the care recipients and those of the caregivers. The ZBI scores of the caregivers were well correlated with the GDS-15 scores of both the care givers and recipients (Spearman's $\rho = 0.492$, $p < 0.001$; $r = 0.250$, $p < 0.001$, respectively). In addition, positive correlations were found between the caregivers' GDS-15 scores and the care recipients' GDS-15 scores (Spearman's $\rho = 0.307$, $p < 0.001$). The correlation between the GDS-15 scores of the caregivers and those of the recipients persisted after adjusting for potential confounders (partial rank correlation coefficient adjusted for the age and gender of care recipients and care givers, caregiver relationship to care recipient, bADL scores and neurodegenerative disorders of recipients, and caregiver's ZBI scores, $r = 0.207$, $p < 0.001$).

Caregivers providing care for recipients who exhibited behavioral problems or neurodegenerative diseases showed significantly higher GDS-15 scores compared with those providing care for recipients who did not exhibit such problems (mean (SD), behavioral problems: presence, 6.24 (3.68), absence, 5.45 (3.78), $p = 0.026$; neurodegenerative disorders: presence, 6.85 (3.69), absence, 5.49 (3.77), $p = 0.011$). However, there were no differences in the caregivers' GDS-15