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TISSUE-SPECIFIC STEM CELLS

STEM CELLS

An aged bone marrow niche restrains rejuvenated hematopoietic stem cells

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Abstract

Aging-associated leukemia and aging-associated immune remodeling are in part caused by aging of hematopoietic stem cells (HSCs). An increase in the activity of the small RhoGTPase cell division control protein 42 (Cdc42) within HSCs causes aging of HSCs. Old HSCs, treated ex vivo with a specific inhibitor of Cdc42 activity termed CASIN, stay rejuvenated upon transplantation into young recipients. We determined in this study the influence of an aged niche on the function of ex vivo rejuvenated old HSCs, as the relative contribution of HSCs intrinsic mechanisms vs extrinsic mechanisms (niche) for aging of HSCs still remain unknown. Our results show that an aged niche restrains the function of ex vivo rejuvenated HSCs, which is at least in part linked to a low level of the cytokine osteopontin found in aged niches. The data imply that sustainable rejuvenation of the function of aged HSCs in vivo will need to address the influence of an aged niche on rejuvenated HSCs.

KEYWORDS
aging, Cdc42, hematopoietic stem cell, niche, osteopontin, rejuvenation

1 | INTRODUCTION

Old hematopoietic stem cells (HSCs) contribute to aging-associated leukemia and aging-associated immune remodeling.[1,2] Aging results in an increase in the number of HSCs in bone marrow (BM), while old HSCs show a reduced reconstitution potential, myeloid skewing, altered gene expression profiles, and epigenetic modifications[3-7] and a switch from asymmetric to symmetric divisions.[8-10] There is also an increase in the frequency of HSCs with an apolar distribution of polarity proteins upon aging.[11,12] Aging of HSCs might even affect lifespan.[13] Mechanisms intrinsic to HSCs as well as extrinsic signals (niche/microenvironment) can cause aging of HSCs.[14,15] For example, reducing the aging-associated increased activity of the small RhoGTPase Cdc42 in old HSCs ex vivo with the specific Cdc42 activity inhibitor CASIN[16] results in rejuvenation of the function of old HSCs. This young-like function of old rejuvenated HSCs remains sustained when they are transplanted into young recipients.[2,9,17]

Young HSCs, when transplanted into an aged BM niche, do show hallmarks of old HSCs.[18,19] This implies a strong influence of an aged niche on the function of HSCs. In line with this concept, we recently demonstrated that a decrease in the level of the secreted cytokine osteopontin (OPN) in the aged BM niche confers hallmarks of aging on young HSCs,[19] like an increase in HSCs pool size (see also Reference 20), a decrease in the frequency of HSCs polar for polarity proteins and an increase in the myeloid differentiation potential of HSCs.[19] Expression of OPN in BM is restricted to the endosteal bone surface.[21] Interestingly, secreted OPN eventually signals via α9β1 integrins on HSCs to regulate Cdc42 activity and thus HSCs polarity.[19,22] The effect of the niche (young or aged) on rejuvenated old HSCs is therefore a critical

question for tailoring approaches to attenuate aging of HSC in vivo. The aim of this study was therefore to determine whether an aged niche might affect the young-like function of rejuvenated old HSCs.

Significance statement

The youthful function of rejuvenated hematopoietic stem cells (HSCs) upon transplantation depends in part on a young niche/microenvironment. The influence of the niche needs therefore to be considered in approaches to rejuvenate old HSCs in vivo for attenuation of aging-associated leukemia or aging-associated immune remodeling.

2 | METHODS

2.1 | Mice

Young C57BL/6 mice (8-10 weeks old, Janvier, St. Berthevin Cedex, France). Aged C57BL/6 mice (19-24 months) and OPN knockout (KO) mice (C57BL/6, 8-12 weeks old) were obtained from the internal stock. C57BL/6.SJL-*Ptprc*^{a}/Boy (BoyJ) young (8-10 weeks) and aged (19-24 months) mice were obtained from Charles River Laboratories, Sulzfeld, Germany, or from the internal stock.

2.2 | Competitive transplantation of LT-HSCs

Two-hundred old LT-HSCs (Ly5.1^{+}) were cultured for 16 hours in HBSS + 10% FBS with or without 5 μM CASIN at 37°C, 5% CO_2, 3% O_2 and transplanted alongside 3×10^5 BM cells from young C57BL/6 mice (Ly5.2^{+}) into C57BL/6 young, aged and young OPN KO recipient mice (Ly5.2^{+}). Donor chimerism in blood and BM was analyzed up to 23 weeks post transplantation. For experiments in Supplementary Figure 1, 200 young or old LT-HSCs (Ly5.1^{+}) were cultured for 16 hours in HBSS + 10% FBS with or without 5 μM CASIN at 37°C, 5% CO_2, 3% O_2 and transplanted alongside 3×10^5 BM cells from young C57BL/6 mice (Ly5.2^{+}) into C57BL/6 young (young into young) or aged (old into aged) recipient mice (Ly5.2^{+}).

2.3 | Flow cytometry and cell sorting

Peripheral blood (PB) and BM cell immunostaining were performed according to standard procedures and analyzed on a LSRII flow cytometer or BD FACSAria III (BD BioSciences, Heidelberg, Germany). Antibodies: anti-Ly5.2 (clone 104), anti-Ly5.1 (clone A20), anti-CD3e (clone 145-2C11), anti-B220 (clone RA3-6B2), anti-Mac-1 (clone M1/70) and anti-Gr-1 (clone RC57BL/6-8C5), anti-CD11b (clone M1/70), anti-B220 (clone RA3-6B2), anti-CD5 (clone 53-7.3), anti-Gr-1 (clone RB6-8C5), anti-Ter119, and anti-CD8a (clone 53-6.7). After lineage depletion by magnetic separation (Dynabeads, Invitrogen, Dreieich, Germany), cells were stained with anti-Sca-1 (cloneD7), anti-c-Kit (clone 2B8), anti-CD34 (clone RAM34), anti-CD127 (clone A7R34), anti CD16/CD32 (clone 2.4G2) (BD Biosciences, Heidelberg, Germany), anti-Flk-2 (clone A2F10), and streptavidin. All antibodies were from eBioscience (Dreieich, Germany), except if stated otherwise. FACS analyses data (stem and progenitor cells) were plotted as a percentage of long-term hematopoietic stem cells (LT-HSCs, gated as LSK CD34$^{-/low}$Flk2^{-}), short-term hematopoietic stem cells (ST-HSCs, gated as LSK CD34^{+}Flk2^{-}), and multipotent progenitors (MPPs, gated as LSK CD34^{+}Flk2^{+}) distribution among donor-derived LSKs (Lin^{neg}c-kit^{+}sca-1^{+} cells). Common myeloid progenitors (CMPs, gated as Lin^{neg}c-kit^{+}CD34^{+}CD16/32^{-}), megakaryocyte-erythrocyte progenitors (MEPs gated as Lin^{neg}c-kit^{+}CD34^{-}CD16/32^{-}), and granulocyte-macrophage progenitors (GMPs gated as Lin^{neg}c-kit^{+}CD34^{+}CD16/32^{+}). FACS analyses data (differentiated cells) are plotted as the percentage of B cells (B220+), T cells (CD3+), and myeloid (Gr-1+, Mac-1+, and Gr-1+Mac-1+) cells among donor-derived Ly5.1 + cells.

2.4 | Immunofluorescence staining

LT-HSCs were seeded on fibronectin-coated glass coverslips, incubated for 16 hours in HBSS 10% FBS, 37°C, 5% CO_2, 3% O_2, with 5uM CASIN, fixed with 4% PFA, washed with PBS, permeabilized with 0.2% Triton X-100 (Sigma, Darmstadt, Germany) in PBS for 20 minutes and blocked with 10% donkey serum (Sigma, Darmstadt, Germany) for 30 minutes. Anti-a-tubulin antibody (Abcam, Berlin, Germany, rat monoclonal ab6160), anti-rat DyLight488-conjugated antibody (Jackson ImmunoResearch, Hamburg, Germany) and anti-H4K16ac (07-329) from Millipore, Darmstadt, Germany, anti-rabbit DyLight549-conjugated antibody (Jackson ImmunoResearch, Hamburg, Germany) for 1 hour at room temperature. Slides were mounted with ProLong Gold Antifade reagent with DAPI (Invitrogen, Molecular Probes, Dreieich, Germany), imaged on an AxioObserver Z1 (Zeiss, Oberkochen, Germany) and analyzed with AxioVision 4.6 software.

2.5 | Statistical analyses

One-way ANOVA or two-way ANOVA were used to compare means among three or more independent groups. Bonferroni post-test to compare all pairs of data set was determined when overall *P*-value was <.05. All statistical analyses were determined with Prism 8.0.1 version.

3 | RESULTS AND DISCUSSION

In previous experiments, young or old Ly5.2 HSCs were transplanted into young Ly5.1 recipients. The Ly5.1 allele in Ly5.1 congenic animals

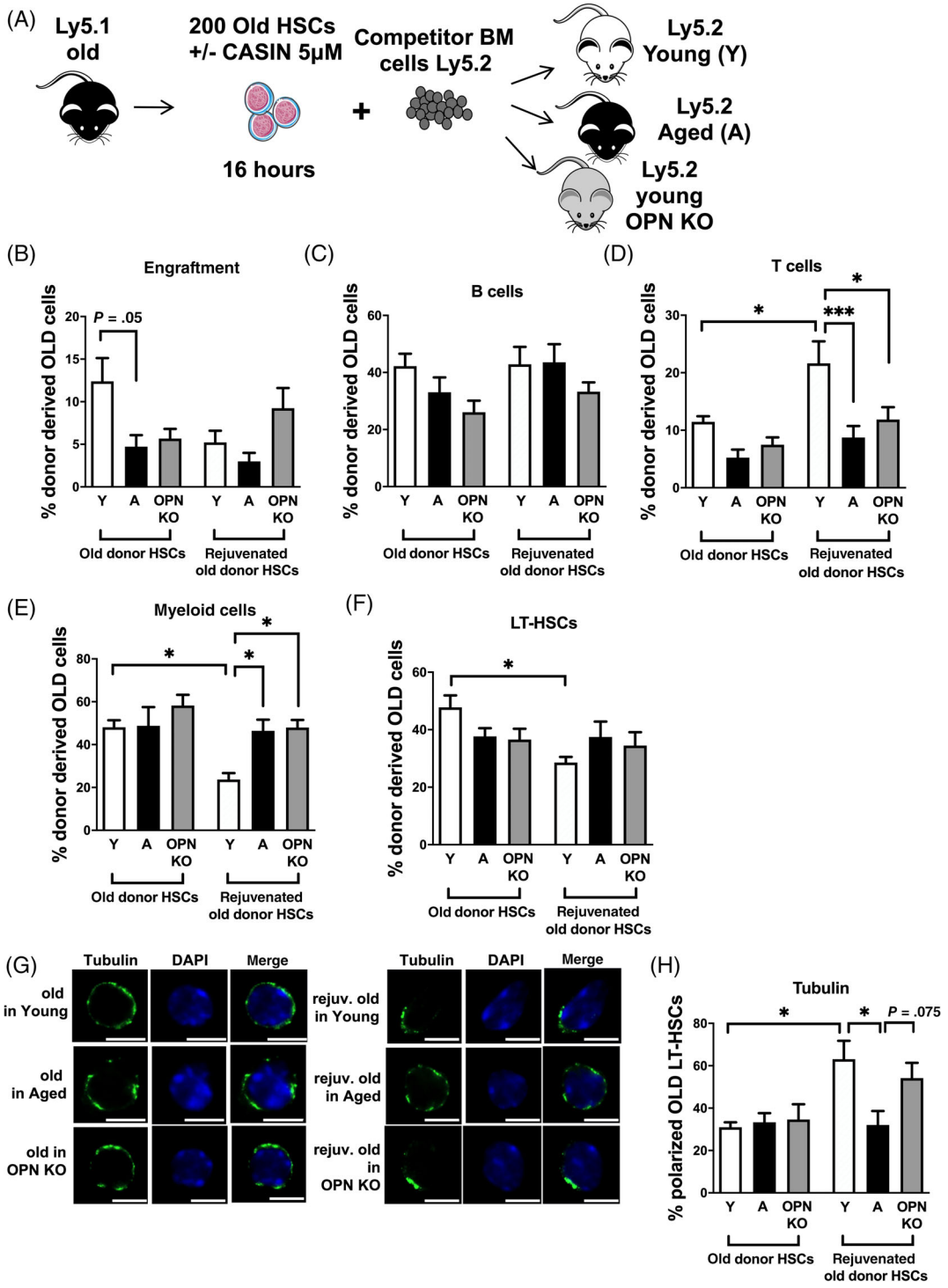


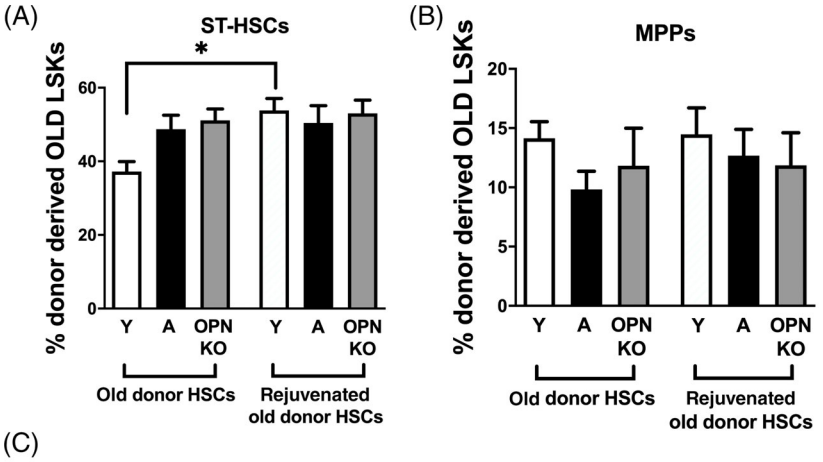
FIGURE 1 An aged microenvironment restrains CASIN-dependent rejuvenation of old hematopoietic stem cells (HSCs). A, Schematic representation of the experimental setup: (Ly5.1+) 200 HSCs cells from old donors were treated +/− CASIN for 16 hours ex vivo and then competitively transplanted into either young (Y), aged (A), or young osteopontin (OPN) knockout (KO) recipient (Ly5.2+) mice. B, Frequency of old donor contribution (Ly5.1+ cells) +/− CASIN to total WBC in peripheral blood (PB) of young, aged and young OPN KO recipient (Ly5.2+) mice. C, Frequency of old B cells +/− CASIN. D, Old T cells +/− CASIN. E, Old myeloid cells +/− CASIN among donor-derived Ly5.1+ cells in PB of young, aged and young OPN KO recipient (Ly5.2+) mice. F, Frequency of old LT-HSCs +/− CASIN in bone marrow (BM) among donor-derived Ly5.1+ LSK cells in young, aged and young OPN KO recipient (Ly5.2+) mice. Data are based on five experimental repeats with three recipient mice per group (eg, n = 11-15 per group). G, Frequency of donor-derived old LT-HSCs +/− CASIN polarized for tubulin sorted from the different experimental groups (Ly5.2+) mice 23 weeks post transplantation. n = 5; ~40 cells scored per sample in each experimental repetition. H, Representative distribution of tubulin (green) in donor-derived old LT-HSCs +/− CASIN (Ly5.1+ cells) sorted from the different experimental groups 23 weeks after transplant. Scale bar = 6 μm. Shown are mean values + 1 SEM. $^{*}P < .05$, $^{***}P < .001$

contains besides Ly5.1 other genes that might be able to influence hematopoiesis.[23] Novel experiments in which young Ly5.1+ HSCs were transplanted into young Ly5.2+ recipients and old Ly5.1+ HSCs transplanted into aged Ly5.2+ recipients confirmed an "aged function" of also old Ly5.1+ HSCs when transplanted into aged Ly5.2+ recipients (Supplementary Figure 1), similar to that previously reported for old Ly5.2 HSCs transplanted into young Ly5.1+ recipients.[17,24] Donor cell engraftment was (as expected) high in Ly5.1+ Y→Ly5.2+ Y (young into young, Y→Y) transplants and low in Ly5.1+ O→Ly5.2+ A (old into aged, O→A) transplants, with a lower contribution of old Ly5.1+ HSCs to B- and T-cells in PB (Supplementary Figure 1B,C). We also observed a higher contribution of old HSCs to the frequency of myeloid cells in PB and to the frequency of LT-HSCs and ST-HSCs in BM (Supplementary Figure 1D-F). In addition, the frequency of MPPs in BM was reduced in O→A compared to Y→Y transplants (Supplementary Figure 1G), whereas frequencies of CMPs, GMPs, and MEPs were similar in both experimental groups (Supplementary Figure 1H-J) and thus not affected by aging. We recently described a reduced frequency of HSCs polar for the distribution of tubulin as a novel hallmark among aged HSCs. HSCs from aged recipients transplanted with old Ly5.1+ HSCs also showed a low frequency of HSCs polar for tubulin (Supplementary Figure 1K,L). The congenic interval that surrounds the Ly5.1 locus therefore does not differentially affect aging-related changes in the phenotypes and the function of old Ly5.1+ HSCs when compared to old Ly5.2+ HSCs. The outcome of these critical control experiments allowed for a comparative analysis of the function of old Ly5.1+ HSCs in distinct types of niches in the following transplantation experiments.

To determine the extent to which the age of the niche contributes to the young-like function and polarity of ex vivo rejuvenated old HSCs, old or old HSCs (Ly5.1+) exposed to the Cdc42 activity inhibitor CASIN (rejuvenated old HSCs[9]) were transplanted alongside Ly5.2 + BM competitor cells into young (as previously published[9]) or in these sets of experiments now also into aged recipients (Figure 1A). In aged mice, the level of the cytokine OPN is strongly reduced in BM resident osteoblasts, whereas a young niche that lacks OPN turns young HSCs functionally old with respect to phenotypes and HSC function.[19,25] Old HSC and rejuvenated old HSCs were therefore also transplanted into OPN KO recipients to test whether lack of OPN in the niche affects the function of old rejuvenated HSCs (Figure 1A). Donor chimerism in blood and BM was analyzed up to 23 weeks post transplantation.

The level of chimerism sustained by old or old rejuvenated HSCs when transplanted into young, aged, or OPN KO recipients was similar for each type of donor HSCs, with the exception of old HSCs transplanted into young recipients. In this setting, and as already[19] reported, old HSCs showed a significantly higher chimerism in comparison to old HSCs transplanted into aged recipients. There was no differential chimerism among young, old, or OPN KO recipients transplanted with old rejuvenated (CASIN treated) HSCs (Figure 1B).

B-cell frequencies in recipients that received old or old rejuvenated HSCs were similar in young, aged, or OPN KO recipients (Figure 1C). The frequency of T cells derived from old rejuvenated HSCs was increased in comparison to the frequency in young recipients transplanted with old HSCs. This frequency was decreased in aged or OPN KO recipients (Figure 1D). This shows that a youthful T-cell frequency (see also Supplementary Figure 1C) supported by old rejuvenated HSCs actually requires a young niche, and this is independent of the age of the thymus (see also Reference 2) and that lack of



(C)

	Young niche	Aged niche
Rejuvenated old HSCs	↑ T cells	↓ T cells
	↓ Myeloid cells	↑ Myeloid cells
	↓ HSC pool size	→ HSC pool size
	↑ H4K16ac polarity	↑ H4K16ac polarity
	↑ Tubulin polarity	↓ Tubulin polarity

FIGURE 2 An aged microenvironment does not impair multipotent progenitors stemming from old rejuvenated hematopoietic stem cells (HSCs). A, Frequency of ST-HSCs and B, Frequency of multipotent progenitors (MPPs) among donor-derived Ly5.1+ LSK cells in bone marrow (BM) of young, aged, and young osteopontin (OPN) knockout (KO) recipient (Ly5.2 +) mice. C, Young-like phenotypes of rejuvenated old HSCs attenuated by an aged niche. Shown are mean values + 1 SEM. *$P < .05$

OPN in in the niche was sufficient to impair the T-cell frequency sustained by old rejuvenated HSCs. The frequency of donor-derived myeloid cells (in % of donor chimerism) was low in young recipients transplanted with old rejuvenated HSCs (Figure 1E and Reference 17). Old rejuvenated HSCs, when transplanted into aged or young OPN KO recipients, returned back to supporting a high level of myeloid cells, similar to the level found in young recipients transplanted with old HSCs (Figure 1E). An aged niche therefore exerts also a strong regulatory influence on the level of myeloid cells in the periphery, independent of the age of HSCs, similarly to what we observed for T cells.

Young recipients that received old rejuvenated HSCs showed a young-like low frequency of LT-HSCs within BM[17] (Figure 1F). Aged or OPN KO animals, when transplanted with old rejuvenated HSCs, showed only a slightly elevated frequency of LT-HSCs (Figure 1F). Old HSC are apolar for the distribution of tubulin or the epigenetic polarity marker H4K16ac, whereas young or old rejuvenated HSCs, when transplanted into young recipients, remain more polar for the distribution of tubulin or H4K16ac[17,19] (Figure 1G,H). Old rejuvenated HSCs, when transplanted into aged recipients, showed a low frequency of cells polarized for tubulin that was similar to that of old HSCs (Figure 1G,H; Supplementary Figure 1K,L). Interestingly, HSCs from OPN KO recipients transplanted with old rejuvenated HSCs presented with a frequency of HSCs polar for the distribution of tubulin that was more similar to the frequency found in young recipients (Figure 1G,H). In contrast, HSCs from aged recipients transplanted with old rejuvenated HSCs, showed a frequency of cells polarized for the epigenetic polarity marker H4K16ac similar to the frequency found in young or OPN KO recipients (Supplementary Figure 1M). These findings imply that while the level of tubulin polarity, but not H4K16ac polarity, is influenced by the aged niche, this effect might not be simply a consequence of a low level of OPN like seen for T-cell differentiation (Figure 1D).

Aging results in a reduced frequency of MPPs in BM, but does not affect the frequency of CMPs, GMPs, and MEPs[17,24] (Supplementary Figure 1G-J). We observed a slight but significant increase in the frequency of ST-HSCs in young recipients which received old rejuvenated HSCs compared to young recipients that received old HSCs (Figure 2A). The frequency of MPPs was interestingly neither affected by rejuvenation of HSCs nor by the type of recipient animal (Figure 2B) nor was by any of the above listed other types of progenitor cells in BM (Supplementary Figure 1N-P).

4 | CONCLUSION

Our data demonstrate that the sustained youthful function of rejuvenated old HSCs upon transplantation into young recipients is at least in part a combination of stem cell intrinsic and stem cell extrinsic contributions (niche/microenvironment). Some of the hallmarks of aging of HSCs that are reverted to a youthful state when rejuvenated old HSC are transplanted into young mice turn back to an aged phenotype when rejuvenated old HSCs are within an aged niche (Figure 2C). This might be, for some of the phenotypes, because of the reduced level of OPN in aged niches. Thus, the influence of the niche on rejuvenated HSCs needs to be taken into account for approaches that aim at rejuvenating old HSCs in vivo.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

N.G.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; G.M., V.S.: collection of data; Y.Z.: data interpretation; M.C.F.: conception and design, data interpretation; H.G.: conception and design, financial support, administrative support, provision of study material, data interpretation, manuscript writing, final approval of manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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