Development of angiotensin II (1-7) analog as an oral therapeutic for the treatment of chemotherapy-induced myelosuppression

Though widely used, cytotoxic chemotherapy is associated with potentially life threatening drug-induced neutropenia, thrombocytopenia, and anemia.[1-3] Beyond the acute dangers of infection, severe fatigue and uncontrolled bleeding, the hematological toxicities associated with these agents may require dosage attenuation or treatment delays. Mitigation of hematological deficits can allow for more dose-dense, on-time, and on-dose administration - a proven strategy for increasing treatment efficacy.[4] Recombinant human granulocyte-colony stimulating factor (rhG-CSF) for neutropenia and erythropoietin (rhEPO) for anemia are now widely used, front-line treatments for chemotherapy-induced myelosuppression.[1,5] As biologics, they are both expensive and require daily injections or the use of cumbersome on-body injectors. Synthetically accessible and orally bioavailable alternatives to these drugs could help to simultaneously decrease the cost of care while improving patient comfort and compliance.

Angiotensin (1-7) (A(1-7)) - the endogenous peptide agonist for the Mas receptor of the renin-angiotensin system (RAS) - plays a well-documented role in stimulating hematopoiesis and accelerating the recovery of circulating cells.[6] A(1-7) has multi-lineage, proliferative effects on early bone marrow progenitors including myeloid

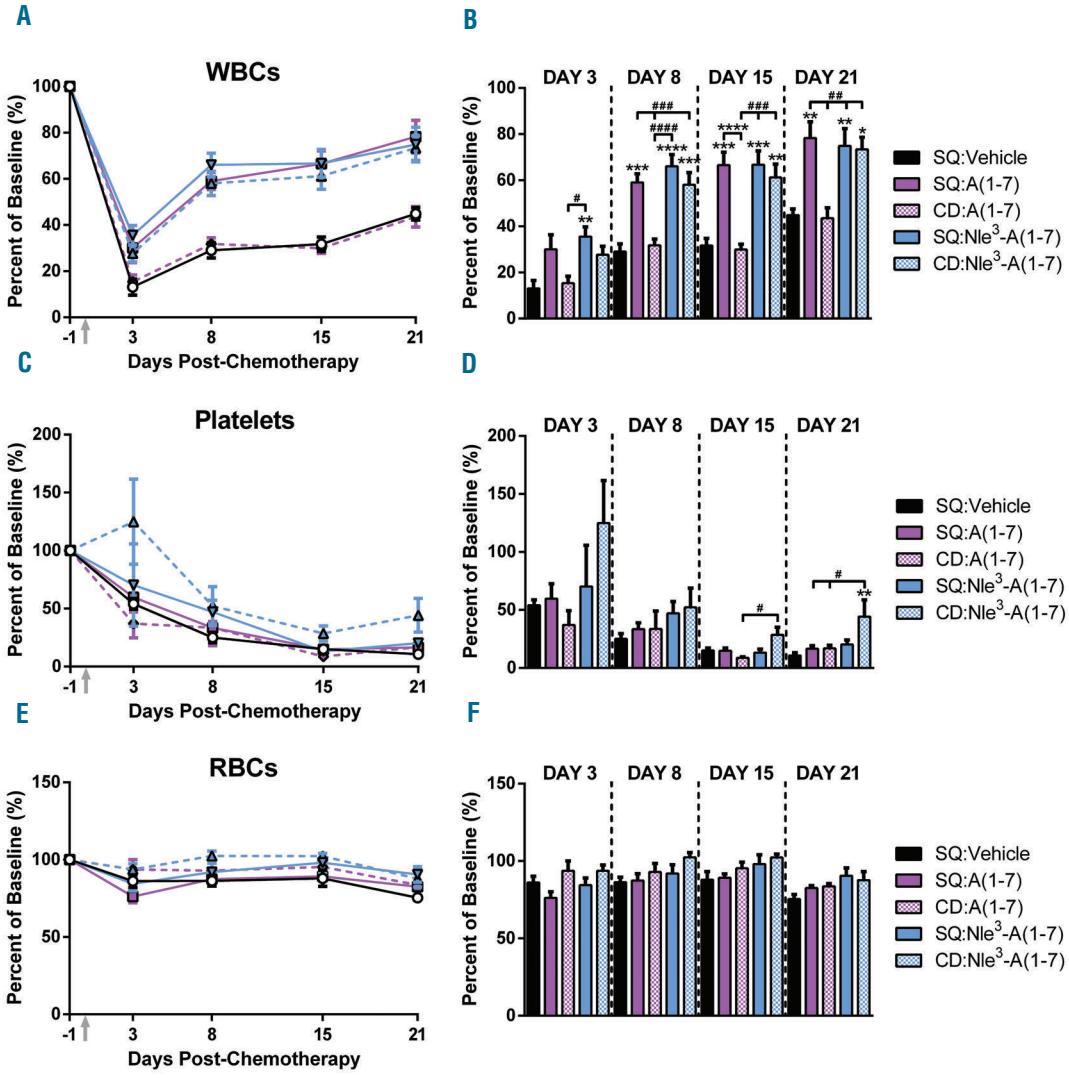

Figure 1. Oral treatment with Nle³-A(1-7) and not A(1-7) reversed leukopenia induced by gemcitabine. Mice (n=7/group in 1 independent experiment) were treated with 160 mg/kg of gemcitabine on day 0 (gray arrow) and treated starting on day 1 through day 21. WBC counts were quantified on days -1 (baseline), 3, 8, 15, and 21. To ease visualization of statistical comparisons, each XY graph is also displayed as a bar graph segmented by day. (A-B) Daily subcutaneous treatment with A(1-7) and Nle³-A(1-7) or oral treatment with Nle³-A(1-7) resulted in a rapid WBC recovery from gemcitabine-induced leukopenia. These treatments showed significantly higher circulating WBCs compared to both SQ vehicle and oral A(1-7) treatment starting on day 8. (C-D) Platelet counts were quantified on days -1 (baseline), 3, 8, 15, and 21. After 21 days of treatment, mice treated daily orally with Nle³-A(1-7) showed a recovery from gemcitabine-induced thrombocytopenia with statistically higher circulating platelets compared to SQ vehicle. (E-F) No effects were seen on RBC counts throughout the study with any treatment. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and ****$P \leq 0.0001$ in comparison with vehicle controls and #$P \leq 0.05$, ##$P \leq 0.01$, ###$P \leq 0.001$, and ####$P \leq 0.0001$ in comparison with oral A(1-7) treatment by one-way analysis of variance (ANOVA). WBC: white blood cell; RBC: red blood cell; SQ: subcutaneous; CD: cyclodextrin.

(colony forming unit (CFU)-GM), megakaryocytic (CFU-MEG), erythroid (burst forming unit (BFU)-E), and common myeloid (CFU-GEMM) progenitor cells and works synergistically with lineage specific growth factors to increase the concentration of circulating formed elements.[7] In a phase I/IIa clinical trial, A(1-7) reduced grade 2-4 anemia, lymphopenia, and thrombocytopenia in breast cancer patients receiving doxorubicin and cyclophosphamide.[8] In a phase IIb study, A(1-7) treatment reduced the incidence and severity of thrombocytopenia in ovarian carcinoma patients receiving a combination of gemcitabine and platinum-based chemotherapy and allowed for an on-time and on-dose delivery of the scheduled chemotherapy dose.[9]

During the development of A(1-7) for hematologic disorders, an analog of A(1-7) substituted at the third position with norleucine (Nle) in place of the valine of A(1-7), Nle^3-A(1-7) was developed for topical use. In a number of studies, Nle^3-A(1-7) displayed a greater ability to stimulate wound healing than A(1-7).[10] While clinical data supports the potential of peptide Mas agonists to stimulate hematopoietic recovery, we sought to develop novel formulations that allow for the oral administration of A(1-7) and Nle^3-A(1-7).

To allow oral dosing treatment while maintaining hematologic potency, we assessed the ability of inclusion complexes of these A(1-7) and Nle^3-A(1-7) to impart oral efficacy. β-cyclodextrins are well known for their capacity to increase the oral bioavailability of peptide therapeutics, including A(1-7).[11] β-cyclodextrin formulations of A(1-7) (β-CD:A(1-7)) and Nle^3-A(1-7) (β-CD:Nle^3-A(1-7)) were prepared by lyophilization.

The efficacy of these formulations was tested in a gemcitabine-induced myelosuppression mouse model in which A(1-7) was previously shown to be effective at restoring circulating white blood cell (WBC) levels.[7] Starting 24 hours after gemcitabine dosing, mice were treated daily by oral gavage with β-CD:A(1-7) or β-CD:Nle^3-A(1-7) or daily by subcutaneous (SQ) injection with A(1-7) (SQ:A(1-7)), Nle^3-A(1-7) (SQ: Nle^3-A(1-7)), or saline (SQ:Vehicle). Both the oral and SQ dose were at 0.3 mg/kg, the dose of A(1-7) used in the phase IIb clinical trial.[9] In addition to day -1 baseline, WBCs, platelets, and red blood cells (RBCs) were quantified on days 3, 8, 15, and 21 in blood samples.

By day 3, the gemcitabine injection produced a marked reduction in circulating WBC (Figure 1A,B) and platelet (Figure 1C,D) levels, though no effects on RBCs (Figure 1E,F) were seen by day 21. Daily SQ treatment with both Mas agonist peptides, A(1-7) and Nle^3-A(1-7), resulted in a significant amelioration of gemcitabine-induced leukopenia (Figure 1A,B).[7] Compared to SQ:Vehicle controls, WBCs were significantly higher with SQ:A(1-7) treatment by day 8 and with SQ:Nle^3-A(1-7) treatment by day 3, an effect that remained robust through day 21. While oral gavage dosing of β-CD:Nle^3-A(1-7) recapitulated the restorative effects of SQ:Nle^3-A(1-7) on WBCs by day 8 and onward, oral β-CD:A(1-7) treatment resulted in WBC counts comparable to the SQ:Vehicle controls (Figure 1A,B). Although β-CD:Nle^3-A(1-7) treatment displayed a day 3 increase in platelets relative to SQ:Vehicle, this only reached statistical significance relative to β-CD:A(1-7) at days 15 and 21 and SQ:Vehicle and SQ:A(1-7) at day 21 (Figure 1C,D). Finally, a comparison

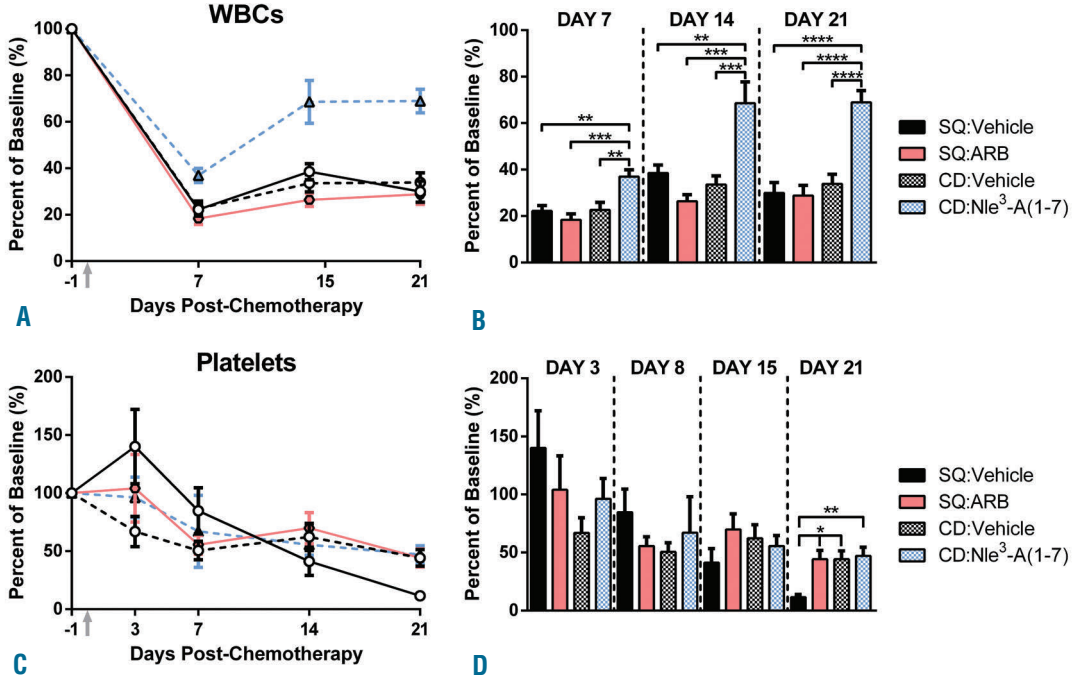

Figure 2. Treatment with Nle^3-A(1-7) and not AT1R antagonist treatment ameliorated gemcitabine-induced leukopenia. Mice (n=7/group in 1 independent experiment) were treated with 160 mg/kg of gemcitabine on day 0 (gray arrow) and treated starting on day 1 through day 21. WBC counts were quantified on days -1 (baseline), 7, 14, and 21. To ease visualization of statistical comparisons, each XY graph is also displayed as a bar graph segmented by day. (A-B) Mice treated daily with oral β-CD:Nle^3-A(1-7) resulted in a rapid recovery in WBCs from gemcitabine-induced leukopenia with statistically higher circulating WBCs compared to both SQ vehicle and oral A(1-7) treatment starting on day 7. Interestingly, treatment with SQ:ARB had no effect on WBCs. (C-D) Platelet counts were quantified on days -1 (baseline), 3, 8, 15, and 21. After 21 days of treatment, mice treated daily orally with Nle^3-A(1-7) showed a recovery from gemcitabine-induced thrombocytopenia with statistically higher circulating platelets compared to SQ vehicle. *P≤0.05, **P≤0.01, ***P≤0.001, and ****P≤0.0001 by one-way analysis of variance (ANOVA). WBC: white blood cell; SQ: subcutaneous; CD: cyclodextrin; ARB: AT1R antagonist.

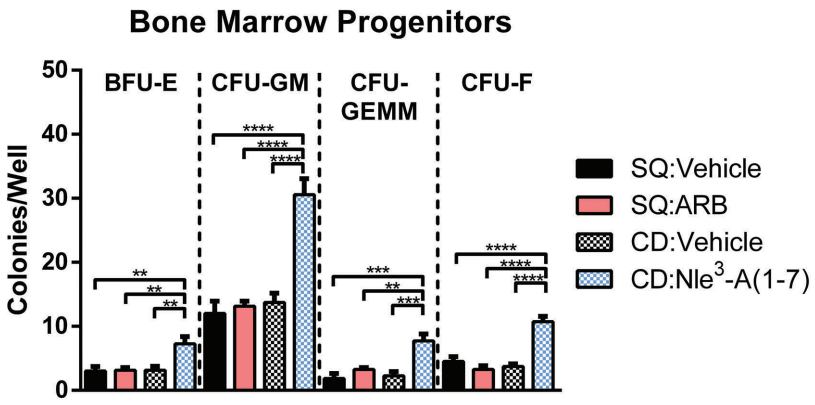

Figure 3. Oral Nle³-A(1-7) treatment significantly increased all hematopoietic lineages measured and MSCs in the bone marrow. Mice (n=7/group in 1 independent experiment) were treated for 21 days beginning 24 hours post-gemcitabine dosing. Bone marrow was harvested at necropsy and bone marrow cells (total nucleated) were cultured in MethoCult™ ($1x10^5$ cells in 900 μL of media) and MesenCult™ ($5x10^5$ cells in 2 mL media) (Stemcell Technologies; Cambridge, MA, USA) to enumerate bone marrow progenitors. In progenitors measured, oral β-CD:Nle³-A(1-7) treatment significantly increased colony counts relative to all other treatments. Interestingly, antagonism of AT1R signaling with SQ:ARB treatment had no effect on MSCs or hematopoietic lineages. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, and $^{****}P \leq 0.0001$ by one-way analysis of variance (ANOVA). SQ: subcutaneous; CD: cyclodextrin; ARB: AT1R antagonist; BFU-E: burst forming unit-erythroid; CFU-GM: colony forming unit-granulocyte, macrophage; CFU-GEMM: colony forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte; CFU-F: colony forming unit-fibroblast.

of the RBC counts for the SQ:Vehicle showed no gemcitabine-induced RBC toxicities as no significant changes were seen in RBCs over the course of the study (Figure 1E,F). Due to its effects on WBCs and platelets, oral β-CD:Nle³-A(1-7) was selected for further study.

In order to validate the utility of activating Mas over blocking the activation of the angiotensin II type 1 receptor (AT1R), the hematological effect of AT1R antagonist (ARB) losartan was assessed relative to Nle³-A(1-7). The same myelosuppression model was used with four treatment groups: SQ:Vehicle, SQ:ARB (10 mg/kg of losartan), oral β-CD:Vehicle, and oral β-CD:Nle³-A(1-7) (0.3 mg/kg). As in the first study, oral β-CD:Nle³-A(1-7) treatment stimulated rapid WBC recovery with significant increases in WBC counts compared to all treatment groups at all time points measured (Figure 2A,B). SQ:ARB treatment resulted in WBC counts equal to or lower than either control arms, SQ:Vehicle and oral β-CD:Vehicle. Though oral β-CD:Nle³-A(1-7) treatment produced significantly higher platelet counts at day 21 compared to SQ:Vehicle, β-CD:Vehicle and SQ:ARB also elevated counts, indicating the effects of treatment on platelets could be reproduced by the ARB or β-CD treatment (Figure 2,D).

To explore the mechanism of β-CD:Nle³-A(1-7) efficacy, the effects of the treatments on stem cell populations in the bone marrow were assessed. The bone marrow harvested at necropsy was cultured in two CFU assays: one to quantify hematopoietic progenitor colonies BFU-E, CFU-GM, and CFU-GEMM and a second to quantify mesenchymal stem cells (MSCs). Across both the hematopoietic lineages measured and MSCs, treatment with oral β-CD:Nle³-A(1-7) increased colony numbers (Figure 3). In contrast, SQ:ARB treatment resulted in progenitor colonies that were comparable to both vehicle treatment groups. These effects on progenitor populations provide a potential mechanism of action of oral β-CD:Nle³-A(1-7) and can potentially account for the differences between the effects of the ARB and Nle³-A(1-7) on WBCs. While these effects on stem cell populations are compelling, further study is needed to assess the effect of Nle³-A(1-7) on neoplastic hematopoiesis and re-treatment toxicity during chemotherapy cycling, though the latter concern is assuaged by the clinical data showing that A(1-7) treatment allowed for the maintenance of a greater chemotherapy dose density than a placebo.[9]

Although A(1-7) has a well-documented ability to treat chemotherapy-induced myelosuppression, thus far the development of Nle³-A(1-7) has focused on its use as a topical agent for healing dermal injuries.[6,8-10] Therefore, in our understanding, this is the first study of Nle³-A(1-7) in this setting. Nle³-A(1-7) has demonstrated equal to or greater efficacy than A(1-7) in a number of models, which suggested that Nle³-A(1-7) would be efficacious *via* parenteral administration. However, the stark contrast between the efficacy of oral β-CD:A(1-7) and β-CD:Nle³-A(1-7) was not predicted. We hypothesize that this physiological difference between orally dosed A(1-7) and Nle³-A(1-7) in β-cyclodextrin results from physiochemical differences between the two peptides. The change from the smaller, bulkier valine of A(1-7) to the more flexible, linear, and hydrophobic norleucine potentially increases the ability of Nle³-A(1-7) to access the β-cyclodextrin cavity and increase van der Waals forces once inside the cavity.[12]

Our new oral β-CD:Nle³-A(1-7) formulation produced comparable effects on WBCs to SQ:A(1-7) and SQ:Nle³-A(1-7). However, oral β-CD:Nle³-A(1-7) was the only agent that produced any significant effects on platelets. As stated previously, in our phase I/IIa and II study, megakaryocytes lineage produced the most robust response to A(1-7) treatment. In our first study, this platelet response was, interestingly, only seen when Nle³-A(1-7) was dosed orally in β-CD, but not when SQ dosed. When repeated with the appropriate β-CD:Vehicle, both β-CD formulated groups, Nle³-A(1-7) and Vehicle, and SQ:ARB produced comparable effects on platelets, demonstrating this effect was not exclusive to Nle³-A(1-7).

Through the use of ARB losartan, we have clearly demonstrated that the hematological effects of A(1-7) and Nle³-A(1-7) on WBCs and progenitor populations cannot be produced by blocking the actions of angiotensin II (A-II) at the AT1R. Previous work in our lab and by other groups have shown the ability of A-II to stimulate hematopoietic proliferation.[13,14] Therefore, through the use of the ARB, losartan, we are blocking the AT1R-mediated beneficial effects of A-II on hematopoiesis, which indicates the importance of A-II/AT1R activation in hematopoiesis and also points to

a unique therapeutic profile of Mas agonists relative to existing RAS-targeting therapeutics.

MSCs were also increased in the bone marrow with β-CD: Nle^3-A(1-7) treatment. While MSCs are well-documented in their capacity to produce a number of different cell types, there is increasing evidence of their role in the bone marrow niche.[15] Therefore, the increase in bone marrow MSCs induced by Nle^3-A(1-7) treatment could either be the result of MSCs protecting and supporting hematopoietic progenitors in the face of chemotherapy, or of MSCs serving as additional evidence of the protective effects of Nle^3-A(1-7) on multiple stem cell lineages.

Finally, in contrast to Nle^3-A(1-7), treatment with the ARB losartan had no progenitor effect, further supporting the functional differences between A-II blockers and Mas agonists. In addition to the well-published ability of Mas agonists to counter-regulate the pathological actions of A-II, they also appear capable of mimicking the beneficial stem cell effects of A-II.[14] Further work to tease out these differences between angiotensin-converting enzyme inhibitors, ARBs, and Mas agonists are of great importance to assist in the clinical development of Mas agonists.

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References

1. Pastor ML, Laffont CM, Gladieff L, Chatelut E, Concordet D. Model-based approach to early predict prolonged high grade neutropenia in carboplatin-treated patients and guide G-CSF prophylactic treatment. Pharm Res. 2015;32(2):654-664.
2. Kuter DJ. Managing thrombocytopenia associated with cancer chemotherapy. Oncology. (Williston Park). 2015;29(4):282-294.
3. Groopman JE, Itri LM. Chemotherapy-induced anemia in adults: incidence and treatment. J Natl Cancer Inst. 1999;91(19):1616-1634.
4. Foote M. The importance of planned dose of chemotherapy on time: do we need to change our clinical practice? Oncologist. 1998;3(5):365-368.
5. John MJ, Jaison V, Jain K, Kakkar N, Jacob JJ. Erythropoietin use and abuse. Indian J Endocrinol Metab. 2012;16(2):220-227.
6. Rodgers KE, Dizerega GS. Contribution of the local RAS to hematopoietic function: a novel therapeutic target. Front Endocrinol (Lausanne). 2013;4:157.
7. Rodgers KE, Espinoza TB, Roda N, Meeks CJ, diZerega GS. Angiotensin-(1-7) synergizes with colony-stimulating factors in hematopoietic recovery. Cancer Chemother Pharmacol. 2013; 72(6):1235-1245.
8. Rodgers KE, Oliver J, diZerega GS. Phase I/II dose escalation study of angiotensin 1-7 [A(1-7)] administered before and after chemotherapy in patients with newly diagnosed breast cancer. Cancer Chemother Pharmacol. 2006;57(5):559-568.
9. Pham H, Schwartz BM, Delmore JE, et al. Pharmacodynamic stimulation of thrombogenesis by angiotensin (1-7) in recurrent ovarian cancer patients receiving gemcitabine and platinum-based chemotherapy. Cancer Chemother Pharmacol. 2013;71(4):965-972.
10. Rodgers KE, Espinoza T, Felix J, Roda N, Maldonado S, diZerega G. Acceleration of healing, reduction of fibrotic scar, and normalization of tissue architecture by an angiotensin analogue, NorLeu3-A(1-7). Plast Reconstr Surg. 2003;111(3):1195-1206.
11. Tiwari G, Tiwari R, Rai AK. Cyclodextrins in delivery systems: Applications. J Pharm Bioallied Sci. 2010;2(2):72-79.
12. Khomutov SM, Sidorov IA, Dovbnya D V, Donova M V. Estimation of cyclodextrin affinity to steroids. J Pharm Pharmacol. 2002; 54(5):617-622.
13. Rodgers KE, Xiong S, Steer R, diZerega GS. Effect of angiotensin II on hematopoietic progenitor cell proliferation. Stem Cells. 2000; 18(4):287-294.
14. Kim S, Zingler M, Harrison JK, et al. Angiotensin II regulation of proliferation, differentiation, and engraftment of hematopoietic stem cells. Hypertension. 2016;67(3):574-584.
15. Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. Nature. 2014;505(7483):327-334.