Dermatopontin Reduces Adhesion of Bone Marrow and Endothelial Cells *in Vitro*

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Dedication

This thesis is dedicated to my parents, who continue to provide their love and support as I further my education.

Abstract

Dermatopontin (DPT) is a small, non-collagenous, and extracellular matrix-associated protein that has not been well characterized. We have previously demonstrated that DPT is secreted by adipocytes in the bone marrow. We have also shown that it can negatively regulate hematopoietic stem cell (HSC) homing and engraftment in mice, but the mechanism by which it does so remains to be elucidated. In this study, we found that DPT reduced adhesion of whole bone marrow (WBM) and endothelial cells in a dose-dependent manner. Interestingly, when WBM cells were incubated with DPT it reduced their ability to adhere on top of endothelial cell monolayers. And using a novel release assay, we also demonstrated that DPT could release WBM cells that were already adhered to the endothelial cells. Based on these studies, we hypothesize that DPT is delaying HSC homing and engraftment by interacting with or disrupting integrin receptors that are critical for transmigration across the endothelial barrier.

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Introduction

Hematopoietic Cell Transplantation

Over the past 50 years, hematopoietic cell transplantation (HCT) has become a well-established treatment option for patients suffering from a variety of hematopoietic disorders. HCT was pioneered in the mid-20th century by Edward Donnall Thomas. He reported the first use of intravenous infusions of bone marrow, following radiation and chemotherapy, to treat patients with cancer[1–3]. Thomas based his early clinical work on evidence that showed mice could survive lethal doses of whole body irradiation if the spleen or bone marrow were shielded[4]. Later evidence indicated that transplanted spleen homogenate or bone marrow could confer protection to irradiation[5–10]. These findings led Thomas to attempt the first human bone marrow transplant (BMT) between identical twins[3,11].

Following the success of Thomas—and a furthered understanding of human leukocyte antigen (HLA)-matching—Robert A. Good successfully performed the first BMT between non-siblings at the University of Minnesota in 1968[12]. This likely solidified the future usefulness of HCT. Today, over 50,000 HCTs are performed annually to treat diseases including acquired hematological malignancies and inherited disorders[13–15]. The work of Thomas and Good paved the way for HCT to become a well-established and life-saving standard of care for patients suffering from diseases and disorders including cancers of the blood and immune system, anemia, immune system deficiencies, and inborn errors of metabolism[11,16,17].

Hematopoietic Stem Cells and the Hematopoietic Niche

HCT is an effective treatment option for hematological diseases and disorders due to unique properties possessed by hematopoietic stem cells (HSCs). HSCs have been studied in depth over the last 50 years and were one of the first stem cells to be characterized[9,10]. Because billions of blood cells must be replenished every day, hematopoiesis is a carefully regulated through an orchestrated process of differentiation and self-renewal. In healthy individuals, the relatively rare population of multipotent HSCs can effectively maintain the entire hematopoietic system. To form all of the major blood cell lineages—erythroid, myeloid, and lymphoid—a limited pool of HSCs[18,19] differentiate into committed progenitor cells, which subsequently terminally differentiate to form every type of blood cell in the body[20]. The HSCs also undergo a tightly regulated self-renewal process in order to maintain the small pool of HSCs[21]. Taking advantage of the self-renewal and differentiation characteristics, HCT is utilized to replace and rebuild a patient's damaged or defective hematopoietic system using HSCs from a healthy donor.

The hematopoietic niche is integral in maintaining and regulating the quiescence, self-renewal, and differentiation of HSCs[22–27]. The niche is a specialized microenvironment located within the bone marrow. It consists of a vascular niche, which regulates differentiation and proliferation as well as an osteoblastic niche which regulates quiescence and self-renewal[23,27–30]. The niche consists of a diverse population of bone marrow cell types including osteoblasts, endothelial cells, and stromal cells[27,29]. These cells provide physical contact and chemical signaling necessary for maintaining and regulating the self-renewal and differentiation characteristics of HSCs[22,23,25,26,31,32].

Importantly, bone marrow cells in the hematopoietic niche also produce chemokines that have been implicated in migration, retention, and homing of HSCs[33–36].

Hematopoietic Stem Cell Homing

HSCs can enter the blood stream via endogenous mobilization[37], induced mobilization by drugs[38], or intravenous delivery during HCT[39]. Once in the peripheral circulation, HSCs undergo active navigation back into the bone marrow, a process that is known as "homing." HSCs naturally home to specific tissues during development; early in development starting in the aorta gonad mesonephros (AGM) region and homing to the fetal liver and then from the fetal liver to the bone marrow as development progresses. HSC migration and homing in the adult organism play an important role in bone marrow homeostasis[33]. Stress signals or radiation- or chemotherapy-induced tissue damage can also cause HSCs to mobilize and re-home to the bone marrow[40,41]. HCT takes advantage of the homing capability of HSCs because transplanted cells will home to the bone marrow in response to a chemoattractant gradient and tissue damage (caused by total body irradiation, or TBI, and/or chemotherapy), much like the homeostatic process[33,42,43].

The homing mechanism utilized by HSCs is a complex, multi-step process that is not really understood. To attract circulating HSCs to the bone marrow, cells in the hematopoietic niche secrete chemokines and other factors. This establishes a chemoattractive gradient in which the hematopoietic niche has relatively high concentration of signaling molecules while the peripheral blood does not. Stromal derived factor-1 (SDF-1; CXCL12) and its receptor, CXCR4, have been implicated as the

main chemoattractive axis in HSC migration, retention, and homing[36,44]. Secretion of SDF-1—by stromal cells, mesenchymal stem cells, perivascular cells, and osteoblasts that are located in the bone marrow[33,34,36,45]—can be induced by bone marrow tissue damage or other stress signals[41,46,47]. The CXCR4 receptor is constitutively expressed on HSCs and can be upregulated in response to stem cell factor (SCF) and hypoxia inducible factor-1 (HIF-1), which are also secreted by cells in the hematopoietic niche[33]. This dynamic expression of SDF-1/CXCR4 allows the hematopoietic niche to signal the mobilized HSCs and initiate the homing process, which occurs relatively rapidly.

In addition to acting as a chemoattractant, SDF-1 also stimulates the upregulation of adhesion molecules required for HSC transendothelial migration through the bone marrow endothelial barrier[43]. Similar to leukocyte extravasation, once mobilized HSCs enter the microvasculature of the bone marrow they are prompted to undergo rolling adhesion that is mediated by P- and E-selectin molecules on the bone marrow endothelial cells (BMECs)[48,49] (**Figure 1A**). Further cytokine and juxtacrine signaling initiates the tight adhesion of HSCs to the BMECs. This tight adhesion relies on the binding of the integrin receptor very late antigen-4 (VLA-4) to vascular cell adhesion protein 1 (VCAM-1) and intercellular adhesion molecules 1 (ICAM-1) engagement with lymphocyte function-associated antigen 1 (LFA-1)[35] (**Figure 1B**). Both VLA-4 and LFA-1 are upregulated by SDF-1 stimulation prior to the HSC-BMEC adhesion[33]. The tightly adhered HSCs then extravasate and enter the bone marrow (**Figure 1C**). This extravasation (also known as diapedesis) is also mediated by VLA-4 and -5, which are

thought to pull the HSCs along the extracellular matrix (ECM) protein fibronectin to enter the bone marrow proper[35,50].

Following successful transendothelial migration, HSCs then go through a process known as lodgment. Lodgment occurs briefly as HSCs are traversing the marrow, migrating to bone marrow regions specific to the hematopoietic niche (**Figure 1D**). Transmembrane-bound SCF (tm-SCF)[51], hyaluronic acid (HA)[52], and osteopontin (OPN)[53] have all been implicated in lodgment. However, due to the lack of a suitable *in situ* models, difficulties in labeling and tracking individual or small populations of cells in the bone marrow and a large functional overlap between adhesion molecules and receptors, a detailed understanding of the lodgment process is not readily obtainable[54].

In the context of HCT, transplanted HSCs that have successfully traversed the bone marrow during lodgment then undergo engraftment in the hematopoietic niche (**Figure 1D**). Successful engraftment also depends upon cell-cell contact and cytokine signaling[54], much like homing and lodgment. Unlike homing and lodgment, however, successful engraftment requires cell division; once in the hematopoietic niche, transplanted HSCs will begin proliferating and differentiating[33]. Donor-derived short-term HSCs (ST-HSCs) and other hematopoietic progenitors serve as the primary source of blood cells early after transplantation (weeks to months). Stable re-establishment of the hematopoietic system occurs with the engraftment of donor-derived long-term HSCs (LT-HSCs)[33,55]. A significant delay or inhibition of the homing process impedes successful engraftment of LT-HSCs and will ultimately lead to graft failure.

Figure 1: Summary of adhesive interactions between HSCs and BMECs during homing.

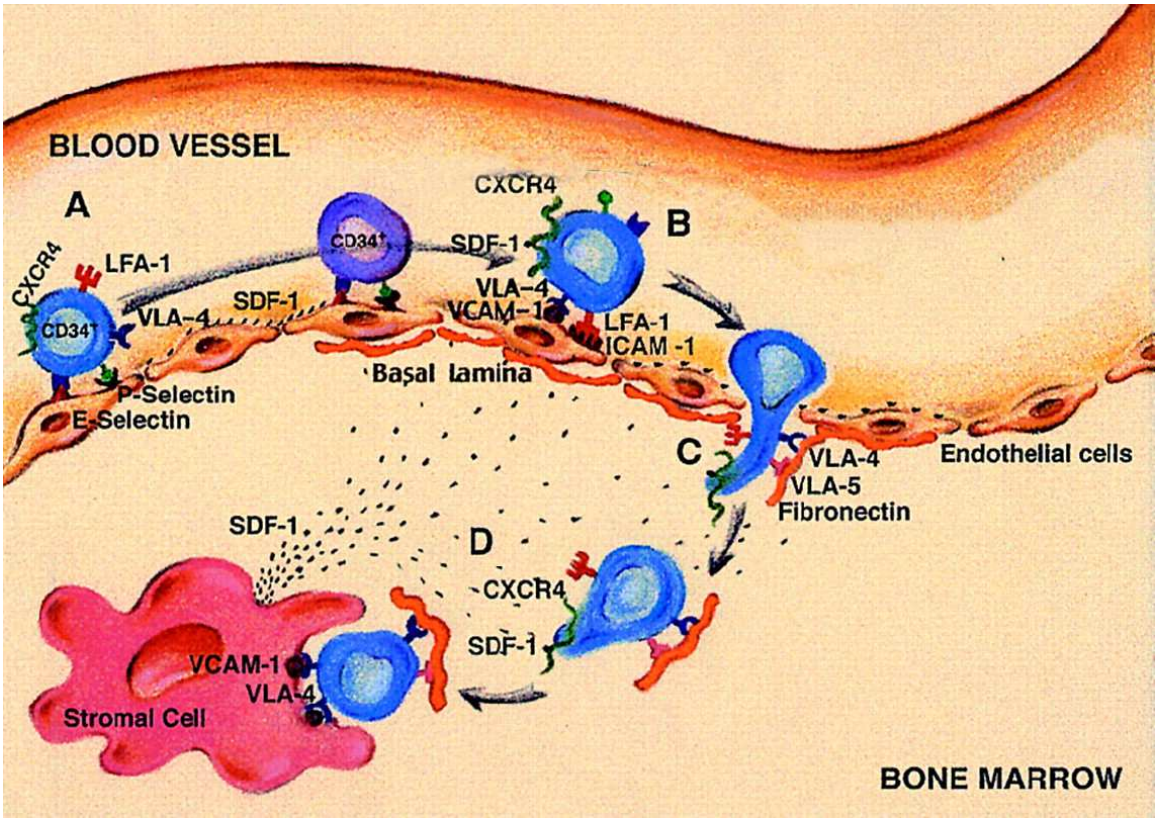

Figure 1: This figure was adapted from Peled et al.[35] Stromal cells in the bone marrow secrete SDF-1, creating a chemoattractive gradient that recruits circulating HSCs to the bone marrow. HSCs bear the SDF-1-specific receptor, CXCR4. (A) Rolling adhesion is initiated by P- and E-selectins on the surface of BMECs. (B) Tight adhesion occurs when VLA-4-bearing HSCs bind to VCAM-1 on BMECs. ICAM-1 also engages LFA-1 during tight adhesion. (C) Transendothelial migration (diapedesis) follows tight adhesion and is mediated by VLA-4 and -5 which pull the HSCs along the ECM protein fibronectin. (D) HSCs then traverse the bone marrow to regions of the hematopoietic niche, where they will interact closely with various support cells. Migration through the bone marrow is known as lodgment and engraftment occurs when HSCs enter the niche and begin proliferation and differentiation. BMECs: bone marrow endothelial cells; HSCs: hematopoietic stem cells; ICAM-1: intracellular adhesion molecule-1; LFA-1: lymphocyte function-associated antigen 1; SDF-1: stromal derived factor-1; VCAM-1: vascular cell adhesion molecule-1; VLA: very late antigen.

Homing-Associated Graft Failure

Despite the success of HCT over the past 50 years, the procedure is still associated with significant morbidity and mortality[11]. GVHD, disease relapse, infection, and graft failure all represent major obstacles for HCT success. Of these, graft failure is one of the most fatal HCT complications and has an incidence rate of up to 5% in the case of allogeneic-HCT[56–60]. It may be difficult to pinpoint the exact cause of graft failure as a number of factors can ultimately cause graft failure during HCT. First, if an inadequate number of donor-derived HSCs are transplanted into a patient, there may not be enough HSCs present to establish a functioning hematopoietic system; such is commonly the case for umbilical cord-derived HSCs[57]. Second, immunologic destruction (host versus graft) likely accounts for a vast majority of graft failures[61] and is mediated by persistent endogenous immune cells[56]. The immune-mediated destruction of transplanted HSCs occurs as early as homing and lodgment processes, preventing engraftment from even occurring. A third point of failure is GVHD, in which immune cells within the transplanted tissue react against the host's body. And finally, it is hypothesized that irradiation- or chemotherapy-induced tissue damage in the bone marrow may induce certain resident bone marrow cells, such as adipocytes, to secrete factors that may delay or impede successful HSC homing or proliferation[53,62,63].

Role of Adipocytes in the Hematopoietic Niche

Adipocytes, like endothelial cells and other stromal cells, are present in and around the hematopoietic niche in the bone marrow[64]. While a vast array of research has

gone into understanding how hematopoietic niche cells regulate and influence hematopoiesis and HSC homing and engraftment[65], the definitive role of adipocytes in these processes remains unclear. A thorough study by Naveiras et al. found that bone marrow taken from adipocyte-rich bones had an overall reduction in the number of hematopoietic progenitor cells and that HCT into genetically fatless (A-ZIP/F1) mice lead to an enhanced recovery of the hematopoietic system[66]. This indicates that HSCs more readily engraft in the absence of adipocyte infiltration into the hematopoietic niche, which occurs with age or after irradiation or chemotherapy. Intriguingly, Naveiras et al. also reported that adipocytes could reduce *in vitro* expansion of hematopoietic cells, likely via secreted, diffusible inhibitors[66]. This finding was reinforced by additional reports showing the adipocyte-secreted factors neuropillin-1[67], lipocalin 2[68], adiponectin[63], and tumor necrosis factor (TNF)-α[69] are each capable of suppressing hematopoietic proliferation. However, the exact adipocyte-derived mediators causing reduction in homing or engraftment efficiency have not been identified.

Preliminary data gathered by our lab (all unpublished data) supports the hypothesis that adipocyte-rich regions of the bone marrow recruit and engraft hematopoietic cells less efficiently when compared to adipocyte-poor regions. In support of Naveiras et al., we found that donor-derived murine HSCs prefer to home to the proximal half of the tibia, which is an adipose-poor region of bone marrow in mice. To identify possible adipocyte-secreted signaling factors (adipokines) that may account for the disparity between HSC homing to adipocyte-rich bone marrow versus adipocyte-poor bone marrow, we performed gene expression analysis on populations of murine

adipocytes and $CD45^+$ hematopoietic cells. Nineteen genes encoding for chemokines, cytokines, or extracellular factors were expressed at least 2-fold higher in adipocytes versus the $CD45^+$ hematopoietic cells. Of these genes, we chose to focus on the small, secreted protein dermatopontin (DPT). DPT had high expression in adipocytes and was shown to be localized to adipocyte-rich areas in the bone marrow. Like other chemokines that affect hematopoietic cell behavior, DPT was highly induced in the bone marrow after irradiation and plasma levels of DPT were elevated for 1-2 days post-radiation. Unlike the other adipokines discussed previously, DPT does not appear to inhibit cell proliferation or differentiation, as shown using colony forming unit (CFU) assays. However, it was revealed that DPT can significantly impair homing and engraftment if mice were pre-treated with a bolus of 2 μg of DPT 30 to 60 minutes prior to HCT. CFU assays done on peripheral blood following transplant revealed a higher number of CFUs were present in the blood compared to untreated mice, indicating that transplanted HSCs were "stuck" in circulation. Together, these preliminary findings (all unpublished data) indicate that DPT is secreted by adipocytes, both naturally and in response to radiation-induced injury, and that the secreted DPT negatively modulates HSC homing and engraftment. The exact mechanism by which DPT regulates these processes is unknown.

Dermatopontin

DPT, also known as tyrosine-rich acidic matrix protein (TRAMP), is a 22 kDa ECM-associated protein that was first discovered in bovine fetal skin[70]. DPT is

composed of 183 amino acids, of which 20 are tyrosine residues. The protein structure consists of five loops, four of which interestingly contain the same repeating residue sequence D-R-E/Q-W-X-F/Y. However, the significance of the loop structure or residue sequence has not been demonstrated. Another residue sequence of note is N-Y-D[71], which may have lysyl oxidase activity that can cross-link collagen[72] among other proteins, allowing DPT to modify the ECM. And finally, DPT contains a candidate integrin-binding residue sequence, R-G-A-T[71,73], indicating the DPT molecule likely interacts with integrin receptors, as has been shown by several studies[74–80].

Unlike other non-collagenous ECM proteins, DPT has not been extensively characterized. In addition to bovine, DPT expression was found to be conserved amongst other mammals[81], including humans[82]. Expression has also been documented in zebrafish[83], birds[81], and invertebrates such as crabs and mollusks[84]. This indicates DPT may have a critical function in the body. In addition to the skin, DPT is widely distributed throughout the kidney, liver, heart, eye, muscle, bone matrix and brain[71,83,85–88]. Whether or not DPT has a physiological role in the hematopoietic system or bone marrow remains to be observed.

Previous studies have shown that DPT interacts with a number of other ECM proteins and can modify their biological activity, hinting at a possible physiological role for DPT. Because DPT was discovered by co-purification along with decorin[70], there is a clear relationship between the decorin and DPT molecules, which later evidence supported[71,89]. *In vitro* studies have revealed that DPT interacts with fibrin, fibrinogen, and fibronectin, promoting fibrillogenesis and enhancing their biological activity[77–79].

Additionally, a comprehensive study revealed that targeted disruption of DPT using dermatopontin-null mice caused abnormal collagen fibrillogenesis, a decrease in the collagen content of skin, an increase in skin elasticity, and a thinner dermis[90]. A separate study using the same dermatopontin-null mouse found defects in collagen fibrils and a decrease in the thickness of the corneal stroma[86,91]. Overall, dermatopontin-null mice were mostly normal although the hematopoietic system was not examined. And furthermore, a small number of studies have revealed that DPT can enhance or inhibit the biological activity of signaling pathways or secreted signaling factors[88,89,92]. Taken together, these studies reveal that DPT can regulate the biological activity of numerous other ECM components and may have an influence on cellular signaling networks.

Since the ECM is a critical component involved in cell adhesion, DPT likely plays a major role in regulating the adhesive properties of cells in the body. DPT also appears to be involved in the response to injury because it is upregulated after irradiation. Supporting this fact, DPT was observed to promote migration and adhesion of epidermal keratinocytes (HaCaT cells)[74] as well as cardiac fibroblasts[75] *in vitro*. DPT expression has also been found in the infarct zone following heart attack in rats[93] and the wound fluid in skin after injury[78]. Additional reports demonstrated that DPT increases adhesion of epithelial (HT1080)[78] and cancer cells[80] *in vitro*. Because it can alter cell adhesion *in vitro*, DPT has been hypothesized to modulate adhesive properties of cells *in vivo*, playing an important role in the wound healing process[71]. This is of interest since radiation and chemotherapy preparative regimens that precede HCT cause significant damage to body tissue, particularly the bone marrow vasculature and stroma.

As previously mentioned, preliminary data obtained by our lab has shown that adipocytes in the bone marrow secrete DPT, especially in response to fatal injury caused by lethal irradiation. Mice pre-treated with DPT prior to HCT showed significantly reduced homing and engraftment of transplanted WBM. These data indicated that DPT is a negative regulator of homing and engraftment, which has never been demonstrated of an adipokine. If DPT is playing a crucial role in bone marrow wound healing—by altering the adhesive properties of cells—it may be inadvertently preventing efficient homing and engraftment of HSCs. However, due to the lack of studies involving DPT in the context of the hematopoietic system, the exact mechanism by which DPT affects the homing and engraftment processes remains uncertain. **We hypothesized that dermatopontin can alter adhesive properties of the extracellular matrix or block integrin receptors on endothelial and bone marrow cells, thereby negatively regulating the homing and engraftment processes.**

To address this hypothesis, we performed adhesion assays to determine how DPT affected WBM and endothelial cell adhesion to plastic and other ECM proteins *in vitro*. Because DPT may reduce WBM cell adhesion to vasculature, we also performed *in vitro* adhesion assays and measured the effect of DPT on WBM cell adhesion to endothelial monolayers. *In vitro* transwell migration assays were utilized to determine if DPT affected WBM cell homing through endothelial monolayers as well. And finally, candidate integrin receptors thought to bind DPT were assessed using flow cytometry. WBM cells were used in these experiments rather than a more defined population of hematopoietic cells because WBM better represents what patients would receive when

undergoing BMT. There is also evidence that donor-derived stromal cells from the hematopoietic niche are important for successful engraftment after BMT[94,95]. It is possible that DPT negatively regulates the homing and engraftment processes by interacting with these beneficial stromal cells. Therefore, we utilized WBM in our experiments as it contains both HSCs and stromal support cells important for HCT.

Materials and Methods

Antibodies and Reagents

Anti-integrin α3 antibodies (#sc-6587) and anti-integrin β1 antibodies (sc-6622) were purchased from Santa Cruz Biotechnology (Dallas, Texas). Anti-integrin α5 antibodies were purchased from BioLegend (#103807; San Diego, CA).

Human fibronectin was purchased Sigma-Aldrich (#F2006; St. Louis, MO). Human osteonectin was purchased from EMC Millipore (#499250; Villerica, MA). Human osteopontin (#1433-OP-050/CF), human vitronectin (#2349-VN-100), pre-coated vitronectin plates (#CWP003), recombinant mouse decorin (#1060-DE-100), recombinant mouse dermatopontin (rDPT; #5749-DP), and recombinant mouse sdf-1α (#460-SD-010) were all purchased from R & D Systems (Minneapolis, MN). Retronectin was purchased from Takara (#T100A; Mountain View, CA). TNF-α was purchased from Peprotech (#300-01A; Rocky Hill, NJ).

Cell Culture

Endothelial cell lines were cultured and used for a variety of *in vitro* adhesion assays and release assays. We chose to test the effect of DPT on endothelial cell adhesion since the bone marrow vasculature represents a major obstacle to homing HSCs. Endothelial cells were also used to create monolayers in transwell migration baskets to create an endothelial barrier for migrating WBM cells. And flow cytometry was utilized to determine if integrin antibodies could inhibit DPT from binding endothelial cells as we hypothesized that DPT may be coating the vasculature in the bone marrow.

The human umbilical vein endothelial cell (HUVEC) line was purchased from Lonza (#C2519A; Basel, Switzerland) and cultured as a monolayer at 37°C. HUVECs were maintained in Endothelial Growth Medium™-2 BulletKit™ (EGM-2) media (#CC-3162; Lonza) and passaged or used for experimentation when 80-90% confluent. HUVEC media was changed every two days.

The human dermal microvasculature endothelial cell (HDMEC) line was purchased from ScienCell (#2000; Carlsbad, CA) and cultured as a monolayer at 37°C. As per supplier recommendation, HDMECs were cultured in endothelial cell medium (ECM) which was also purchased from ScienCell (#1001). The ECM media was supplemented with 25 mL FBS, 5mL endothelial growth supplement, and 5 mL penicillin/streptomycin solution, all of which came with the media kit. ECM media was replaced every two days, as directed, and cells were passaged when 80-90% confluent.

The VeraVec™ mouse bone marrow endothelial cell (BMEC) line was purchased from Angiocrine Bioscience (#mVera-bon-01; New York, NY) and cultured as a monolayer at 37°C in an incubator with 5% CO_2/5% O_2. BMECs were maintained in a complete mouse endothelial cell media which consisted of advanced DMEM/F12 media (#12634-010; Life Technologies, Carlsbad, CA), 50 µg/mL endothelial cell supplement (#J64516; Alfa Aesar; Heysham, United Kingdom), 20% FBS, 1% antibiotic-antimycotic solution (#15240-061; Invitrogen, Carlsbad, CA), 10 mM HEPES buffer (#25-060-Cl; Mediatech, Inc.; Herndon, VA), 5 µM SB431542 small molecule (#1614; R & D Systems), 50 µg/mL heparin (#25021-400-10; Sagent Pharmaceuticals; Schaumburn, IL), 1X Glutamax™ solution (#35050-061; Life Technologies), 1% non-essential amino acids

(#11140-050; Life Technologies), 20 ng/mL fibroblast growth factor-2 (FGF-2; #100-18B, Peprotech), and 10 ng/mL vascular endothelial growth factor (VEGF; #450-32; Peprotech). Because FGF-2 and VEGF degrade in solution, they were added directly to the culture flask whenever the media was changed. Media change occurred every two days and BMECs were passaged when 100% confluent, as suggested by supplier.

Human mesenchymal stem cells (huMSCs) were cultured in MSC media which contained minimal essential medium (MEM)-alpha, 10% FBS, 1% Glutamax, and 1% penicillin/streptomycin (#15070-063; Life Technologies). MSC media was changed every two days and huMSCs were passaged when 80-90% confluent.

Murine Bone Marrow Isolation

Whole bone marrow (WBM) was isolated for use in a variety of *in vitro* adhesion assays, release assays, and migration assays. We chose to use WBM in these assays instead of a more defined population of HSCs. This was done because patients undergoing BMT receive WBM during the transplant opposed to receiving only hematopoietic cells, so we wanted our assays to better represent the population of cells that would be transplanted under normal conditions.

Young mice were euthanized using CO_2 and subsequently perfused with 10 mL of PBS. Long bones from the hind legs were dissected out and cleared of muscle and connective tissue. Bone marrow was harvested by crushing the dissected bones in a pestle containing 5 mL PBS + 2% fetal bovine serum (FBS; referred to as PBS+). Supernatant was filtered through a 40 μm nylon filter and placed on ice. Remaining bone fragments were incubated with 5 mL of Accutase (#07920; StemCell Technologies;

Vancouver, Canada) for 10 minutes at 37°C to release remaining bone marrow cells. After incubation with Accutase, bone marrow fragments were diluted and washed three times using 5 mL cold PBS and filtered through a 40 μm filter into the primary filtrate. The cell suspension was then spun down at 600g for 5 minutes at 4°C. Red blood cells were then lysed with 1X RBC Lysis Buffer (#420301; BioLegend), according to manufacturer's recommendations. WBM cells were then washed twice with PBS+ and used directly for experimentation or further enrichment.

Sca1$^+$ Enrichment

Sca1$^+$ cells were enriched from isolated WBM for use in determining how DPT dosage affected hematopoietic cell adhesion *in vitro*. We enriched for Sca1$^+$ cells because they represent a more refined population of hematopoietic cells located within WBM. Sca1$^+$ cell enrichment was performed by magnetic separation using the EasySep™ Mouse Sca-1 Positive Selection Kit (#18756; StemCell Technologies) according to manufacturer's recommendations. Following enrichment, Sca1$^+$ cells were suspended in PBS+.

Zebrafish Whole Kidney Marrow Isolation

Whole kidney marrow (WKM) as isolated from zebrafish for use in adhesion assays. WKM was used because we wanted to assess whether or not DPT affected adhesion of cells from a non-mammal animal.

Zebrafish were euthanized by rapid cooling in 4°C water. Kidneys were dissected as previously described[96]. Briefly, forceps were used to tease the kidney tissue away from the body, starting from the anterior end and working towards the posterior. The

dissected kidneys were then placed in PBS+ and thoroughly homogenized by rapid pipetting. Suspension was filtered through a 40 μm filter and the cells were spun down at 500g for 5 minutes at 4°C. Whole kidney marrow (WKM) cells were washed three times with PBS+.

CellTracker™ Green CMFDA Staining

To fluorescently label cells for use in adhesion, release, and migration assays, CellTracker™ Green CMFDA dye (CT Green; #C2925; Life Technologies) was utilized according to manufacturer's recommendations. Briefly, after harvesting cells they were re-suspended in 10 mL of PBS+ containing 2.5 μM CellTracker™ Green CMFDA dye. Cells were stained for 30-60 minutes at 37°C in an incubator. After staining, cells were allowed to recover in 10 mL of appropriate media for 30-45 minutes at 37°C. Cells were washed twice with PBS+, placed on ice, and used directly in experimentation.

A CT Green lysis buffer was formulated as a 10X solution containing 5% Triton™ X-100 (#X100; Sigma-Aldrich) dissolved in Hank's balanced salt solution (HBSS) without phenol red (#14025092; Life Technologies). Lysis buffer was further diluted 1:10 in HBSS just prior to use.

ECM Adhesion Assays

To determine how well WBM cells adhere to various ECM proteins, wells of a black, 96-well tissue culture plate were coated with 20 μg/mL decorin, fibronectin, osteonectin, osteopontin, retronectin, or vitronectin overnight at 4°C. Negative control wells were coated with 100 μL PBS. The next day, wells were blocked at room temperature for two hours with a solution of 5% bovine serum albumin (BSA; #A2153;

Sigma-Aldrich) in PBS. During blocking, BSA binds to any plastic that was not bound by the previous proteins. After blocking, wells were washed twice with PBS. CT Green-stained WBM cells were suspended in PBS+ and inoculated into each well at a density of 1.5×10^5 cells/well. Cells were allowed to adhere for 1 hour at 37°C in an incubator. After incubation, wells were carefully washed three times with PBS. Cells were then lysed with 100 µL of 1X CT Green lysis buffer. Fluorescence intensity was measured with a BioTek® Synergy 2 (BioTek; Winooski, VT) plate reader using a 485 nm excitation laser.

We utilized a similar adhesion assay to determine how DPT affects WBM cell adhesion to wells that were instead coated with a cocktail of the ECM proteins. Wells were instead coated with a protein cocktail containing decorin, fibronectin, osteonectin, osteopontin, and vitronectin; each at a concentration of 50 µg/mL. Total coating volume was increased to 250 µL instead of 100 µL. ECM cocktails that received DPT were inoculated with a total of 4 µg of DPT just after other ECM molecules were added. Wells that did not receive DPT were instead inoculated with an equivalent volume of PBS. DPT was thoroughly mixed in to the cocktail via pipetting.

Cell Adhesion to Plastic

We hypothesized that DPT reduced the overall adhesiveness of cells and therefore that cell adhesion to plastic would be reduced in the presence of DPT. To test this, we conducted very fundamental adhesion assays, in which WBM cells or endothelial cells were incubated with DPT and allowed to adhere to a tissue culture plate.

Assays involving cell adhesion to plastic were conducted as follows: CT Green-stained cells were suspended in RPMI media 1640 (#32404-014; Life Technologies). DPT was added to the RPMI media at a final concentration of 36 µg/mL. Control samples received an equivalent volume of PBS instead. Cells were allowed to incubate with DPT for 3 minutes at room temperature. The wells of a black, 96-well plate were then seeded at a density of 5 x 10^4 cells/well. Cells were allowed to adhere for 1 hour at 37°C in an incubator. Following incubation, wells were carefully washed once with PBS and lysed with 100 µL of 1X CT Green lysis buffer. Fluorescence intensity was measured with a BioTek® Synergy 2 plate reader using a 485 nm excitation laser.

In experiments with TNF-α activation of endothelial cells, TNF-α was added to culture media at a concentration of 2 ng/mL the day before the experiment.

Aorta Immunohistochemistry

To determine if DPT binds to vasculature, we dissected aortas, incubated them with DPT and performed immunohistochemistry. Aortas were carefully dissected from mice and rinsed with PBS. One end of the aorta was sutured shut. The aorta was then filled with a 0.5 µg/µL DPT solution (~5 µL) and clamped shut. It should be noted that this is in excess of physiological concentrations, which are measured in the range of pictograms per milliliter after irradiation. The aortas were then incubated at 37°C for 1 hour. Following incubation, the DPT solution was removed and the aortas were washed with PBS. They were then frozen in Tissue-Tek® optimal cutting temperature (O.C.T.) compound (#4583; Sakura, Torrance, CA) and sent to the Histology and Microscopy Core Facility at the University of Minnesota for processing and immunohistochemistry

(IHC). IHC against DPT was performed using an anti-6X His tag® antibody (#ab9108; Abcam, Cambridge, MA).

Cell Adhesion Assays

DPT has been associated with numerous ECM proteins and has been shown to modulate their biological activity. We hypothesized that DPT may be affecting cell adhesion by interacting with ECM proteins important for the adhesion process. To test this hypothesis, we performed adhesion assays in which WBM, WKM, or endothelial cells were allowed to attach to fibronectin- or vitronectin-coated tissue culture plates in the presence or absence of DPT.

WBM Adhesion to Vitronectin

For the experiment involving WBM cell adhesion to vitronectin, pre-coated vitronectin plates purchased from R & D Systems were used. CT Green-stained WBM cells were suspended in PBS+ and inoculated into each well at a density of 1.5×10^5 cells per well. Wells that received DPT were inoculated with a total of 2 µg of DPT along with the cells. Wells lacking DPT were inoculated with an equivalent volume of PBS. Cells were allowed to adhere at 37°C for 1 hour. After incubation, wells were carefully washed three times with PBS. Cells were then lysed with 100 µL of 1X CT Green lysis buffer. Fluorescence intensity was measured with a BioTek® Synergy 2 plate reader using a 485 nm excitation laser.

WKM Adhesion to Fibronectin

Wells of a black, 96-well plate were coated with 20 µg/mL fibronectin overnight at 4°C. The next day, wells were blocked with a solution of 1% BSA in PBS for 1 hour

at room temperature. Wells were then washed once with PBS. CT Green-stained WKM cells were suspended in PBS+. DPT was added to cells at a final concentration of 20 μg/mL. Control samples received an equivalent volume of PBS instead. A black, 96-well plate was then seeded with the cells at a density of 3×10^4 cells/well. Cells were allowed to adhere for 2 hours at 37°C in an incubator. Following incubation, wells were washed twice with PBS and lysed with 100 μL of 1X CT Green lysis buffer. Fluorescence intensity was measured with a BioTek® Synergy 2 plate reader using a 485 nm excitation laser.

HUVEC Adhesion to Vitronectin

A pre-coated vitronectin plate was used. CT Green-stained HUVECs were suspended in PBS+ and inoculated into each well at a density of 3×10^4 cells/well. Wells that received DPT were inoculated with a total of 1 μg of DPT along with the cells. Cells were allowed to adhere at 37°C for 2 hours. After incubation, wells were carefully washed twice with PBS. Cells were then lysed with 100 μL of 1X CT Green lysis buffer. Fluorescence intensity was measured with a BioTek® Synergy 2 plate reader using a 485 nm excitation laser.

HDMEC Adhesion to Fibronectin

Wells of a black, 96-well plate were coated with 20 μg/mL fibronectin ± 1 μg DPT overnight at 4°C. CT Green-stained HDMECs were suspended in ECM media and inoculated on to the late at a density of 1.5×10^5 cells/well. Cells were allowed to adhere overnight in a 37°C incubator. The next day, wells were gently washed with PBS and

were then lysed with 100 µL of 1X CT Green lysis buffer. Fluorescence intensity was measured with a BioTek® Synergy 2 plate reader using a 485 nm excitation laser.

Dosage Assays

We wanted to know if DPT affected cell adhesion in a dose-dependent manner. To test this, we assayed the adhesion of various cell types to wells coating with increasing concentrations of DPT.

Wells of a black, 96-well plate were coated with the indicated concentrations of DPT overnight at 4°C. The next day, wells were blocked with a solution of 1% BSA in PBS for 1 hour at room temperature. Wells were then washed with PBS. Cells were inoculated on to the plate at a density of 3×10^4 cells/well. In the case of the HDMECs, the cell density was 1.5×10^5 cells/well. Plate was incubated at 37°C for 1-3 hours. Following incubation, wells were washed once with warm PBS. Cells were then lysed with 100 µL of 1X CT Green lysis buffer. Fluorescence intensity was measured with a BioTek® Synergy 2 plate reader using a 485 nm excitation laser.

In the case of the vitronectin-coated plate, 5×10^4 CT Green-stained WBM cells were inoculated into each well. DPT was added to the cells up to the final concentrations specified. Wells were mixed via pipette before incubation and plate was incubated at 37°C for 2 hours. After incubation, wells were washed twice with PBS. Cells were then lysed with 100 µL of 1X CT Green lysis buffer.

WBM Adhesion to Endothelial Cells

HSCs undergoing homing have to first adhere to endothelial cells in the bone marrow before entering the hematopoietic niche. Cells failing to adhere to the

vasculature will not be able to properly home to the niche. We next wanted to determine if DPT affected the adhesion of WBM cells to endothelial cells. To assay this, we cultured monolayers of endothelial cells and allowed WBM cells to adhere in the presence or absence of DPT.

Monolayers of HDMECs were cultured in the wells of black, 96-well plates until confluent. On the day prior to the experiment they were activated with TNF-α. CT Green-stained WBM cells were suspended in RPMI and incubated with ~20 μg/mL DPT for 3 minutes prior to plating. Cells were inoculated on top of the HDMECs at a density of 1 x 10^5 WBM cells/well. Cells were allowed to adhere for 1 hour at 37°C. Following incubation, wells were carefully washed once with warm PBS and cells were then lysed with 100 μL of 1X CT Green lysis buffer. Fluorescence intensity was measured with a BioTek® Synergy 2 plate reader using a 485 nm excitation laser.

Release Assay

After HSCs adhere to the endothelial cells in the bone marrow they need to maintain stable adhesion in order to undergo transendothelial migration. We wondered if DPT was able to release WBM cells that were already adhered to endothelial cell monolayers. To test this, we allowed WBM cells to adhere to endothelial monolayers, after which the cultures were incubated with DPT. WBM cells that released from the endothelial monolayers were then measured.

Monolayers of HDMECs were cultured in the wells of a 96-well plate until confluent. CT Green-stained WBM cells were suspended in StemSpan™ media (#09650; StemCell Technologies). WBM cells were inoculated on to the HDMECs at a density of

2 x 10^5 WBM cells per well. WBM cells were then allowed to adhere to the endothelial monolayer overnight at 37°C. The following day, wells were washed once with PBS to remove non-adhered cells. Wells were then incubated with DPT at a concentration of 25 µg/mL for 1 hour at 37°C. Wells that did not receive DPT were inoculated with PBS instead. After incubation, 90 µL of the supernatant was transferred to 10 µL of 10X CT Green lysis buffer. Fluorescence intensity was measured with a BioTek® Synergy 2 plate reader using a 485 nm excitation laser.

Transwell Migration Assay

To determine if DPT had an effect on *in vitro* migration of WBM cells, we conducted transwell assays which included an endothelial monolayer that WBM cells had to migrate through.

In this experiment, baskets of a 24-well, 5 µm pore transwell plate (#07200154; Fisher Scientific, Waltham, MA) were coated with 20 µg/mL fibronectin overnight at 4°C. The following day, 5 x 10^4 HUVECs were inoculated into each transwell basket and were cultured at 37°C until confluent. The transwells were then transferred to wells containing 600 µL of RPMI ± 100 ng/mL sdf-1α. CT Green-stained WBM cells were suspended in RPMI and were inoculated into each transwell at a density of 5 x 10^5 cells/ well. Transwells that received DPT were inoculated with 1 µg of DPT, which was thoroughly mixed with the cells. WBM cells were allowed to migrate overnight at 37°C. The following day, 550 µL of the bottom chamber was removed for analysis. 11,000 PKH26 reference microbeads (#P7458; Sigma-Aldrich) and 0.5 µL Sytox Blue were added to each cell solution. The number of WBM cells that migrated through the

transwell was counted using a BD FACSCanto™ RUO Special Order System flow cytometer with the reference beads as a counting gate.

Blocking adhesion With Integrin Antibodies

Because DPT contains amino acid residue sequences predicted to be integrin binding motifs, we hypothesized that DPT may be interacting with or inhibiting integrin receptors important for the HSC homing process. To confirm that specific integrins are important for cell adhesion, we used functional blocking antibodies against anti-integrin α3, α5, and β1.

BMECs were activated with TNF-α, as described previously. Cells were harvested using trypsin, stained with CT Green, and suspended in RPMI. Cells were then incubated with integrin α3 + integrin β1 or integrin α5 + integrin β1 functional blocking antibodies. Integrin α3 and integrin β1 antibodies were used at a dilution of 1:20 while the integrin α5 antibodies were used at a dilution of 1:100. Cells were then inoculated on a black, 96-well plate at a density of 5×10^4 cells per well. Cells were allowed to adhere at 37°C for 1 hour. After incubation wells were washed once with warm PBS and were then lysed with 100 µL of 1X CT Green lysis buffer. Fluorescence intensity was measured with a BioTek® Synergy 2 plate reader using a 485 nm excitation laser.

Flow Cytometry

In order to confirm that DPT is binding to specific integrin receptors we used functional blocking antibodies and measured the number of cells bound with fluorescently labeled DPT. First, we incubated endothelial cells with a combination of functional blocking antibodies to inhibit the integrin receptors VLA-3 and -5.

Fluorescently-labeled DPT was then incubated with the cells and flow cytometry was utilized to measure the number of cells that bound the labeled DPT after integrin blocking.

For this experiment, confluent monolayers of HUVECs were harvested using non-enzymatic dissociation buffer (#C5789; Sigma-Aldrich), according to manufacturer's recommendations. Cells were suspended in MACS buffer (PBS containing 5mg/mL BSA and 2 mM EDTA) and were aliquot into analysis tubes. The cells were then incubated with α3 (1:20), α5 (1:100), or β1 (1:20), integrin blocking antibodies for 30 minutes on ice. After incubation with the antibodies, 1 µL of Alexa Fluor® 488-labled DPT (DPT-488) was added to each of the samples and incubated on ice for 5 minutes. After incubation, the cells were washed once with MACS buffer and re-suspended in 200 µL of MACS buffer for analysis. 0.5 µg of propidium iodide (#P4864; Sigma-Aldrich) was added as a viability dye. Cells were analyzed using a BD FACSCanto™ RUO Special Order System flow cytometer.

In the case of the VLA-3 and -5 blocking experiment, cells were incubated with a combination of integrin α3+β1 antibodies or integrin α5+β1 antibodies on ice for 30 minutes. Integrin α3 and β1 antibodies had been desalted and were used at a dilution of 1:4 while the integrin α5 antibody was used at a dilution of 1:20. Following incubation with antibodies, 1 µL DPT-488 and 1uL 1:10 fixable near-IR dead cell stain (#L10119; Life Technologies) were added to each sample and incubated on ice for 5 minutes. Cells were washed once with MACS buffer and were re-suspended in 300 µL of 2%

paraformaldehyde/PBS solution and were incubated on ice for 15 minutes. Cells were spun down and re-suspended in 200 µL PBS for analysis.

Statistical Analysis

Data was presented as mean ± s.e.m. and analyzed using unpaired *t* test. *P* values less than or equal to 0.05 were considered significant.

Results

Dermatopontin Alters WBM and WKM Adhesion in vitro

Numerous ECM molecules and related integrin receptors have been implicated in HSC homing and maintenance of the hematopoietic niche[97]. We hypothesized that DPT interacts with these ECM proteins and receptors, which blocks HSC homing. Following an extensive literature search, we screened promising protein candidates in cellular adhesion assays to confirm if any play a role in the adhesion of WBM cells (**Figure 2**). ECM molecules screened were decorin, fibronectin, osteonectin, osteopontin, retronectin, and vitronectin. We found that the recombinant protein retronectin, which is an active fragment of fibronectin, significantly increased adhesion of murine WBM cells, as expected[98]. Likewise, the complete fibronectin molecule increased WBM cell adhesion, albeit not as robustly as retronectin. Decorin quite significantly reduced overall adhesion, which seems to agree with some previous studies[99,100]. Osteopontin and osteonectin had little effect on WBM cell adhesion. Interestingly, vitronectin did not show a significant increase in adhesion in this experiment, despite being a well-known cell adhesion molecule (**Figure 2A**). It should be noted that subsequent experimentation found vitronectin indeed promoted WBM cell adhesion (**Figure 2B**). Based on this data decorin, fibronectin, and vitronectin were the focus of future experiments.

The ECM is a complex milieu of proteins and secreted factors, which together form a vast network involved in processes such as structural support, adhesion, and signaling. The exact role of DPT in the ECM is not known, so we wanted to observe how WBM cells adhered to a "matrix" of proteins in the presence or absence of DPT. To do

so, we coated a 96-well plate with a cocktail of decorin, fibronectin, osteonectin, osteopontin, and vitronectin. We found that if DPT was added to the ECM cocktail during protein coating there would be fewer WBM cells that adhered (**Figure 2C**). This contradicts previous studies[74,78], which have shown that DPT can increase adhesion. This suggests that DPT plays a somewhat ambiguous role in the modulation of cellular adhesion and that it may be cell-type specific or dependent on interactions with other molecules present in the ECM.

Figure 2: DPT reduces WBM cell adhesion to ECM coated wells

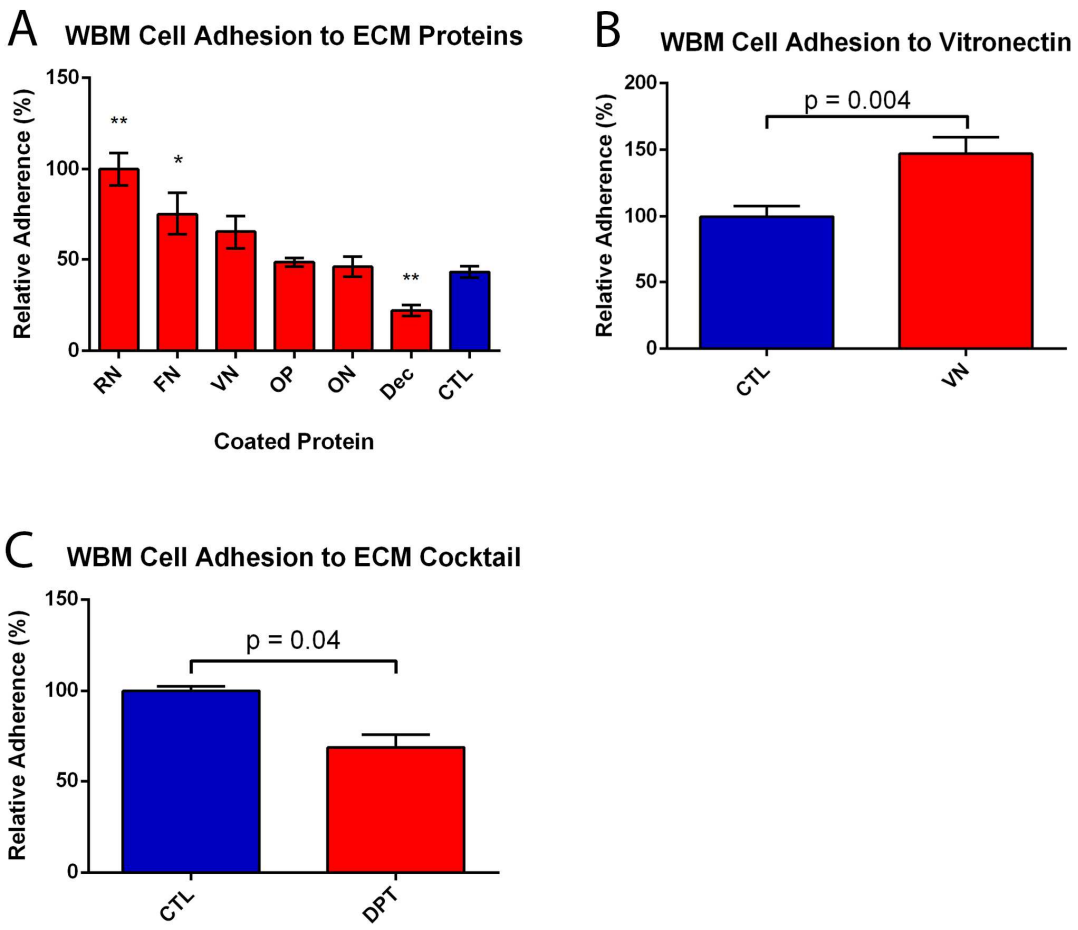

Figure 2: Adhesion of murine WBM cells in wells coated with individual ECM components (A-B) or an ECM cocktail (C). (A) Relative adherence of WBM cells plated in wells coated with Dec, FN, ON, OP, RN, or VN. RN and FN significantly increased WBM cell adhesion while Dec significantly decreased adhesion. (B) Relative adherence of WBM cells plated in wells coated with VN. (C) Relative adherence of WBM cells plated in wells coated with an ECM cocktail (Dec, FN, ON, OP, and VN) ± 4 µg DPT. DPT decreased WBM cell adhesion to this ECM cocktail. ** $P < 0.01$, * $0.01 < P < 0.05$, n=3. CTL: control, Dec: decorin, DPT: dermatopontin, FN: fibronectin, ON: osteonectin, OP: osteopontin, RN: retronectin, VN: vitronectin.

To further characterize DPT in the context of the hematopoietic cells, we performed additional adhesion assays. Adhesion of WBM cells on to vitronectin-coated wells was significantly decreased in the presence of DPT (**Figure 3A**). In a very

fundamental adhesion assay, we examined how DPT affects adhesion of WBM cells to uncoated plastic and found adhesion was drastically reduced when cells were incubated with DPT just prior to inoculation into a 96-well plate (**Figure 3B**), suggesting that DPT is binding to the WBM cells and somehow preventing their attachment to the plastic. And finally, we also observed that DPT could decrease the adhesion of zebrafish WKM cells to FN (**Figure 3C**), indicating that DPT may have a similar biological function in zebrafish. Taken together, these data show that DPT can reduce the adhesiveness of marrow cells, possibly demonstrating an interesting physiological function of DPT. These observations suggest that DPT could act as a negative regulator of WBM cell adhesion *in vivo*, controlling their attachment to other cells or the ECM in the hematopoietic niche.

Figure 3: Dermatopontin reduces overall WBM and WKM cell adhesion *in vitro*

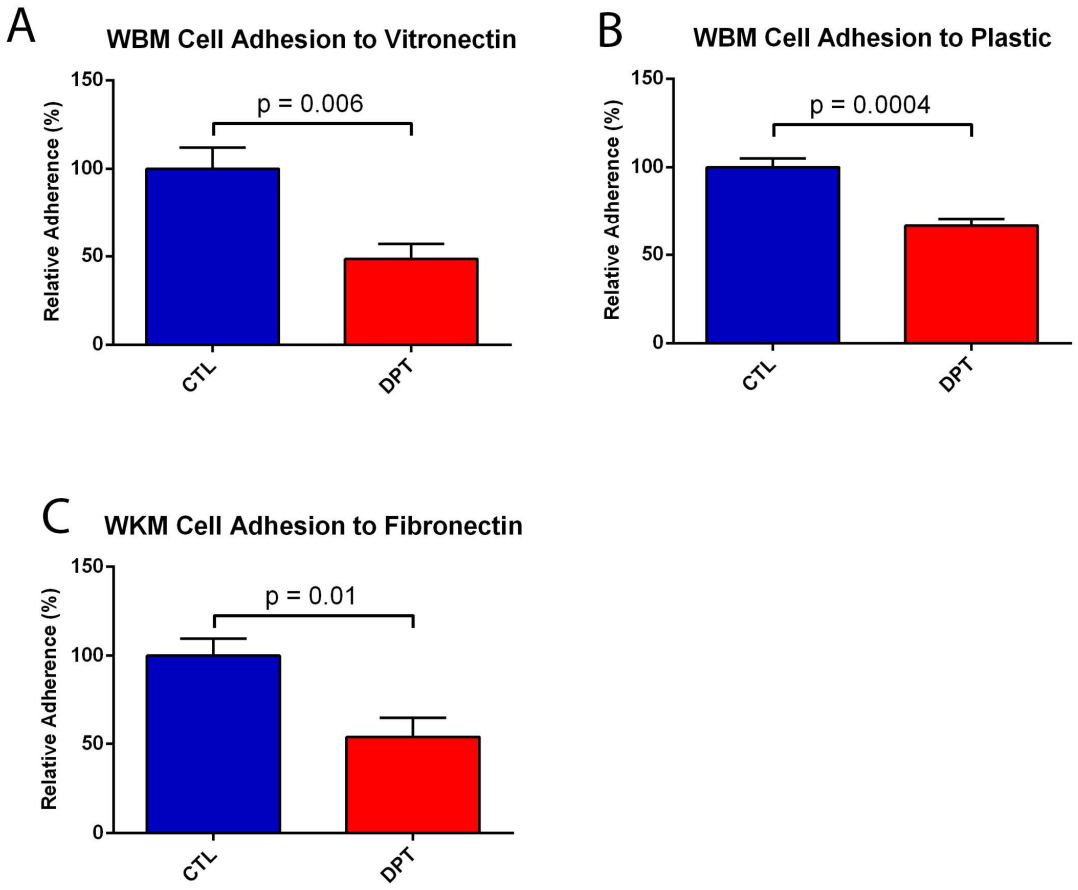

Figure 3: Adhesion of WBM or WKM cells in the presence or absence of DPT. (A) Relative adherence of WBM cells plated in VN-coated wells ± 4 µg DPT. DPT reduced cell adhesion to vitronectin. (B) Adherence of WBM cells to plastic following incubation with 36 µg/mL DPT. This indicates that DPT is binding to WBM cells and preventing their attachment to the plastic. (C) Relative adherence of WKM cells plated in FN-coated wells ± 2 µg DPT. DPT significantly reduced WKM cell adhesion to fibronectin. CTL: control, DPT: dermatopontin, n=6.

Dermatopontin Binds to Endothelial Cells and Alters Cell Adhesion in vitro

We hypothesized that adipocyte-secreted DPT may be interacting with endothelial cells in the bone marrow as well. This interaction may be re-enforcing a physical barrier after radiation and chemotherapy, blocking migrating HSCs. Our lab has previously

demonstrated that HUVECs rapidly take up Alexa Fluor® 488-labled DPT (DPT-488) in culture (unpublished data). To demonstrate that DPT interacts with vasculature, we dissected intact aortas from mice and incubated them with rDPT (**Figure 4**). We used aortas since isolation of capillaries from bone marrow is extremely difficult. Immunohistochemistry (IHC) revealed DPT bound to endothelial cells in the lumen of the aorta (**Figure 4A**), while control aortas incubated with PBS did not reveal any DPT (endogenous DPT is likely too miniscule for detection) (**Figure 4B**). This confirms our previous findings that DPT-488 binds to endothelial cells *in vitro*.

Figure 4: Dermatopontin binds to vasculature *ex vivo*

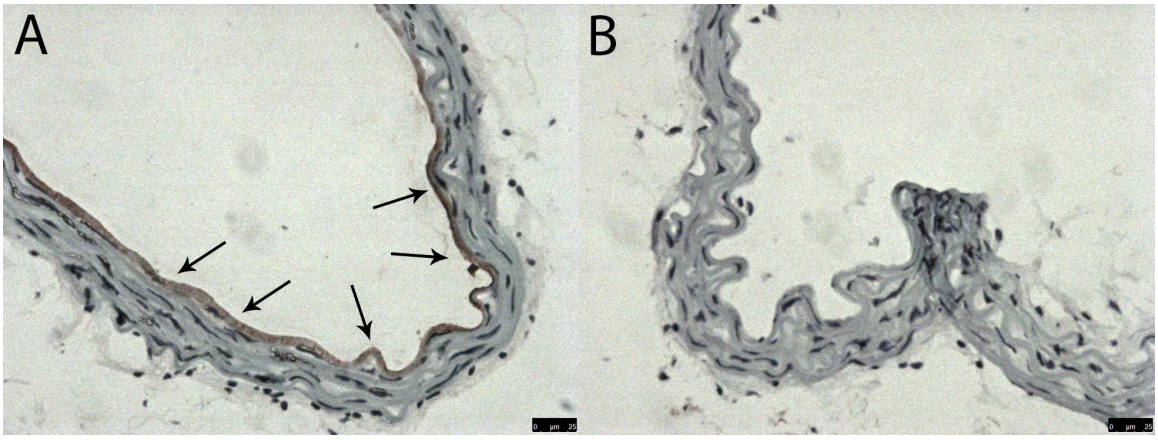

Figure 4: IHC revealing DPT readily binds to endothelial in intact aorta. Aorta were dissected and incubated with (A) and without (B) DPT. IHC was performed against the 6X His-tagged DPT molecule. Arrows indicate brown DPT staining in the lumen of the aorta. 25 µm scale bars.

Having demonstrated DPT can alter WBM and WKM cell adhesion, we predicted it would do the same for endothelial cells. To confirm, we performed adhesion assays using a variety of endothelial cell lines. We found that DPT had no influence on human umbilical vein endothelial cell (HUVEC) cell adhesion to plastic (**Figure 5A**). However,

HUVEC adhesion to vitronectin is significantly decreased with the addition of DPT (**Figure 5B**), supporting the hypothesis that DPT dynamically exerts its influence on cell adhesion and may depend on the presence of other ECM molecules. These findings also corroborate the data showing WBM cell adhesion to vitronectin was also decreased in the presence of DPT.

Figure 5: Dermatopontin decreases HUVEC adhesion to vitronectin

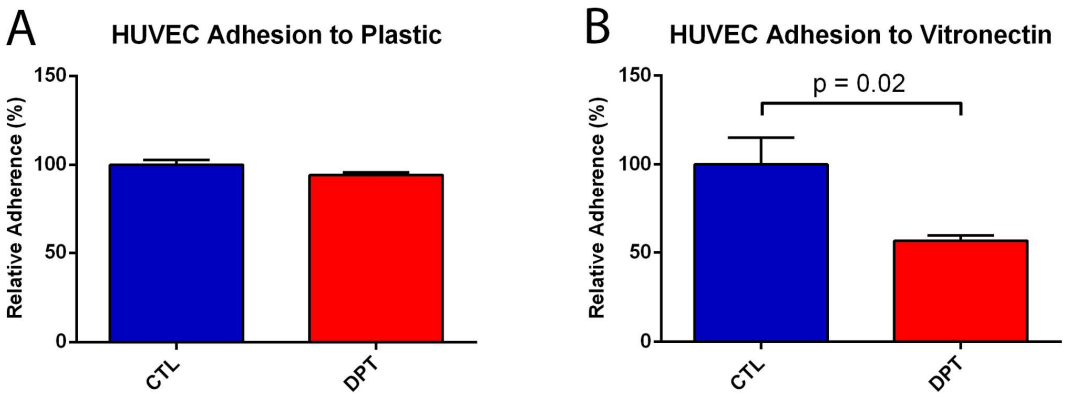

Figure 5: HUVEC adhesion to plastic (A) or vitronectin (B) in the presence or absence of DPT. (A) Relative adherence of HUVECs to plastic wells incubated with 20 µg/mL DPT. HUVECs bound to DPT-coated wells and control wells equally. (B) Relative adherence of HUVECs to a vitronectin-coated plate ± 1 µg DPT. DPT reduced HUVEC adhesion to vitronectin. CTL: control, DPT: dermatopontin, n=6.

In addition to HUVECs, we have also assayed human dermal microvascular endothelial cells (HDMECs) and VeraVec™ mouse bone marrow endothelial cells (BMECs)[101,102]. In agreement with data thus far, DPT decreased adhesion of HDMECs to plastic (**Figure 6A**) and fibronectin (**Figure 6B**). Activation of HDMECs with TNF-α does not substantially change the degree to which DPT decreases adhesion (**Figure 6C**),

indicating DPT is not affecting molecules upregulated by TNF-α in HDMECs. Like the other endothelial cells, adhesion of BMECs to plastic was also significantly diminished upon incubation with DPT (**Figure 7**). Interestingly, DPT had a more substantial effect on the bone marrow endothelial cells compared to the other endothelial cell lines. This indicates that DPT may have a more robust interaction with support cells in the bone marrow.

Altogether, our adhesion assays have consistently shown that DPT negatively influences cell adhesion under numerous conditions, including a variety of endothelial cells, as well as WBM and WKM cell types.

Figure 6: Dermatopontin decreases overall adhesion of HDMECs

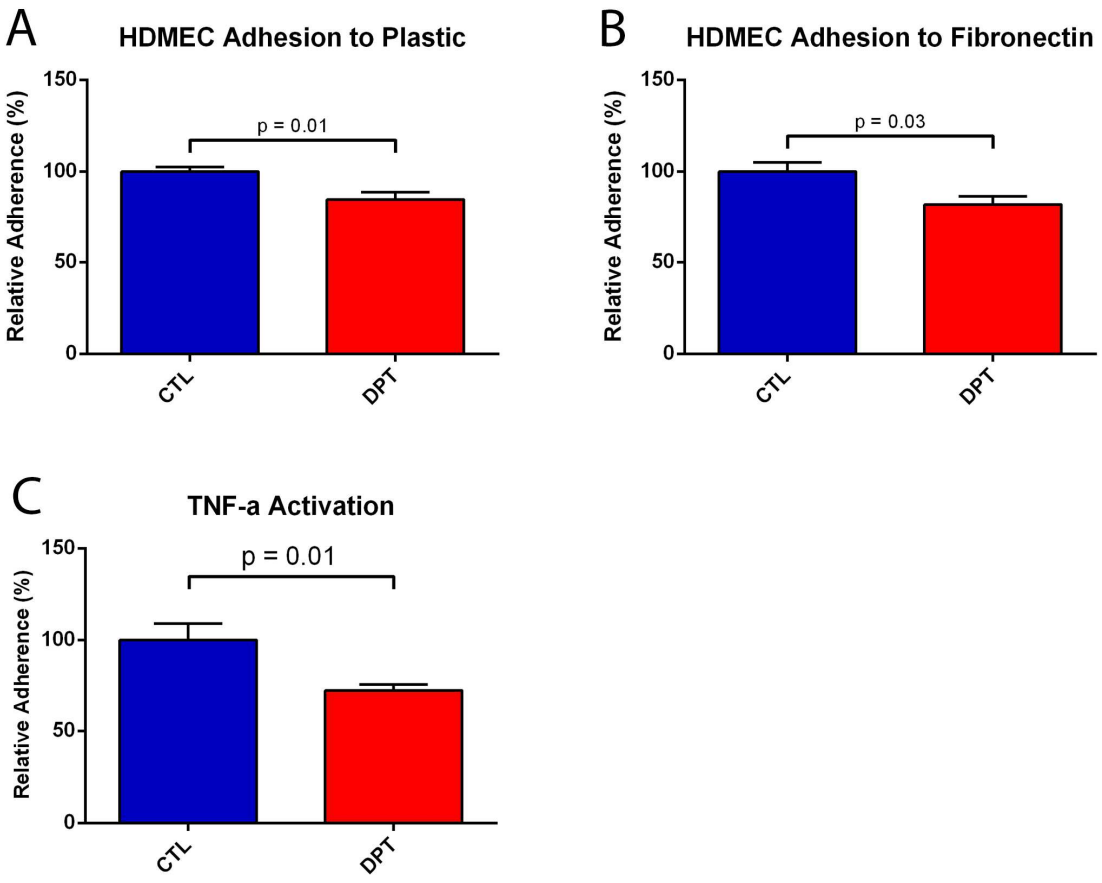

Figure 6: HDMEC adhesion to plastic (A) or fibronectin (B) in the presence or absence of DPT and TNF-α activation (C). (A) Relative adherence of HDMECs to plastic after incubation with 36 µg/mL DPT. DPT decreased cell adhesion as seen previously. (B) Relative adherence of HDMECs to fibronectin-coated wells ± 1 µg DPT. DPT significantly reduced HDMEC adhesion to fibronectin. (C) Effect of TNF-α activation of HDMECs adhering to plastic. TNF-α activation of HDMECs did not change the degree to which DPT alters adhesion. CTL: control, DPT: dermatopontin, HDMEC: human dermal microvascular endothelial cell, TNF-a: tumor necrosis factor-alpha, n=6.

Figure 7: Dermatopontin decreases BMEC adhesion

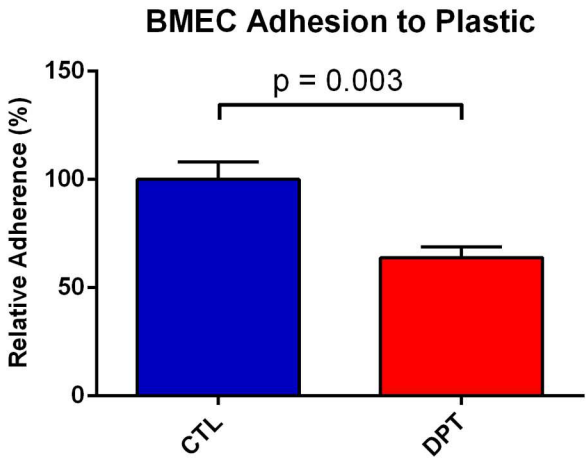

Figure 7: Relative adherence for BMEC adhesion to plastic after incubation with 36 µg/mL DPT. DPT drastically reduced adhesion of BMECs to plastic indicating substantial interaction with DPT compared to other cell types. BMEC: bone marrow endothelial cell, CTL: control, DPT: dermatopontin, n=6.

Loss of Adhesion is Dose-Dependent

We next determined if the loss of cell adhesion was dependent on the amount of DPT added to the system (**Figure 8**). For these experiments we coated wells with increasing concentrations of DPT. In agreement with previous data, we found that DPT reduced adhesion of HDMECs *in vitro*. This effect happened in a dose-dependent manner, with a significant reduction in adhesion starting at a DPT concentration of 10 µg/mL (**Figure 8A**).

Mesenchymal cells are another important cell type present in the hematopoietic niche. Because of this, we were curious if DPT affected adhesion of human mesenchymal stem cells (huMSCs) as well. DPT had a significant effect on huMSC adhesion, in a dose-dependent manner (**Figure 8B**), suggesting the importance of DPT in

regulating the adhesiveness of cell types found in the bone marrow. Further testing revealed that WBM cell adhesion to plastic (**Figure 8C**) and to vitronectin (**Figure 8D**) were both affected by DPT in a dose-dependent manner as well. To determine the dose-response with a more defined population of hematopoietic cells, we performed an adhesion assay using $Sca1^+$ selected hematopoietic cells. Consistent with previous data, we found that DPT affects $Sca1^+$ cell adhesion in a dose-dependent manner (**Figure 8E**). Because $Sca1^+$ cells represent hematopoietic cells in WBM, this indicates that DPT also directly influences adhesion of hematopoietic cells in addition to endothelial cells and other support cells found in the hematopoietic niche.

Figure 8: Loss of cell adhesion is dose-dependent

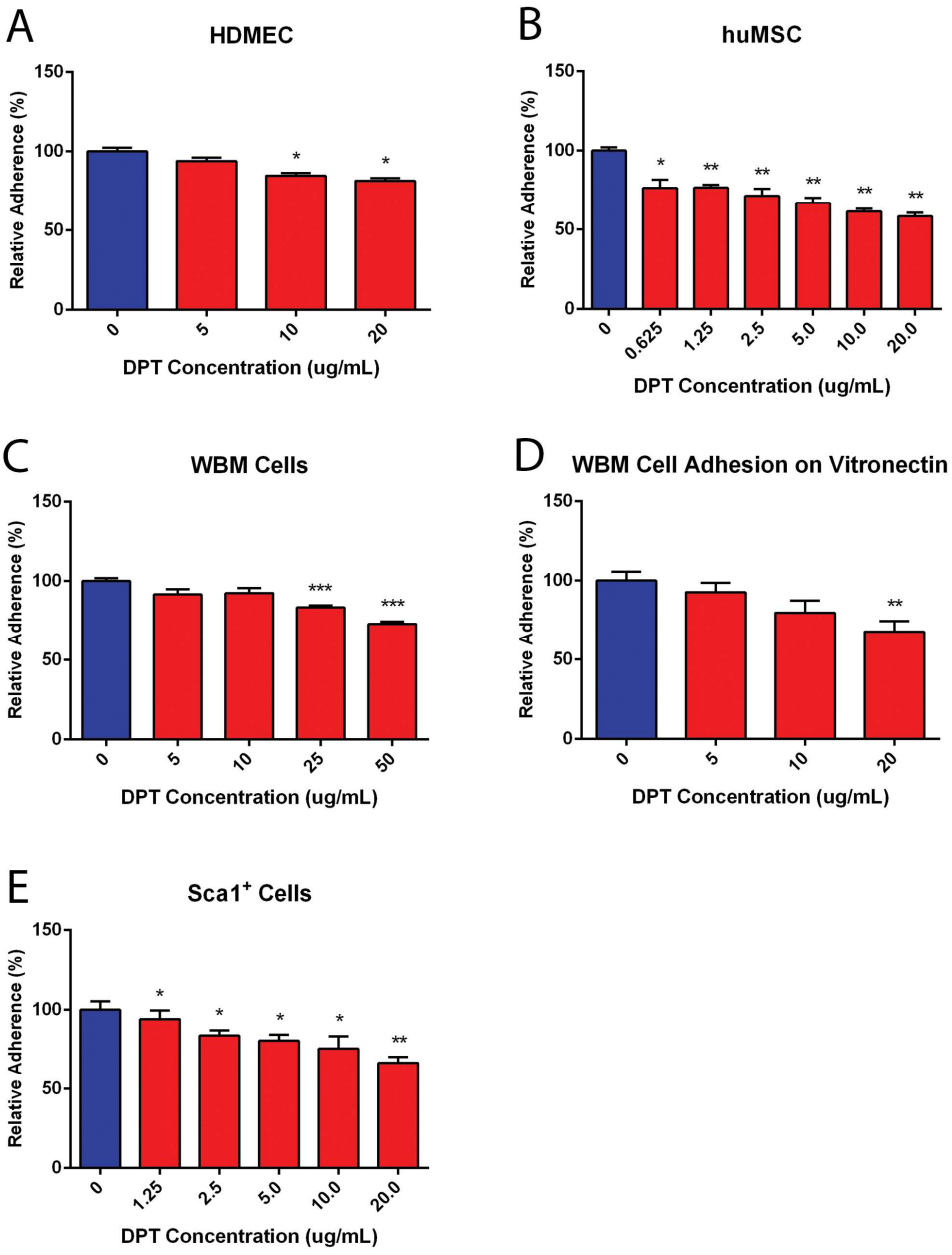

Figure 8: Relative adherence of HDMECs (A), huMSCs (B), WBM cells (C, D), and Sca1$^+$ cells (E) to increasing concentrations of DPT. DPT decreased cell adhesion for each cell type in a dose-dependent manner. *** $P < 0.001$, ** $0.001 < P < 0.01$, * $0.01 < P < 0.05$, n=6. DPT: dermatopontin, HDMEC: human dermal microvascular endothelial cell, huMSCs: human mesenchymal stem cells.

Dermatopontin Reduces WBM Attachment to Endothelial Cells in Vitro

Endothelial cells constitute the first major physical barrier to homing HSCs. In order to undergo extravasation, HSCs need to first undergo rolling adhesion to bind to endothelial cells. This is mediated by P- and E-selectins. Following this, tighter adherence mediated by integrin receptors occurs. We wanted to know if DPT affected WBM (which contains HSCs) adherence to endothelial cells, so we cultured a monolayer of HDMECs and adhered WBM cells on top of them (**Figure 9**). We found that activating the HDMECs with TNF-α lead to an overall increase in the number of adherent WBM cells. This was expected as TNF-α activation of endothelial cells causes the upregulation of selectin molecules and various integrin ligands, which are integral in the initial adherence of HSCs to endothelial cells *in vivo*[103–105]. As hypothesized, significantly fewer WBM cells adhered to activated HDMECs in the presence of DPT. However, it is unknown if this observation is due to a reduction in HSC or stromal cell adhesion to endothelial cells, which will have to be explored further with more defined populations of WBM cells. Because there was not a statistical difference between DPT and control treatments for un-activated HDMECs, the degree to which DPT exerts its influence on adhesion is likely dependent on the relative abundance of other adhesion molecules that are upregulated by TNF-α activation. This implies that the pre-transplant conditioning regimen may be important and required for attachment to occur.

Figure 9: Dermatopontin reduces WBM cell adhesion to endothelial cells

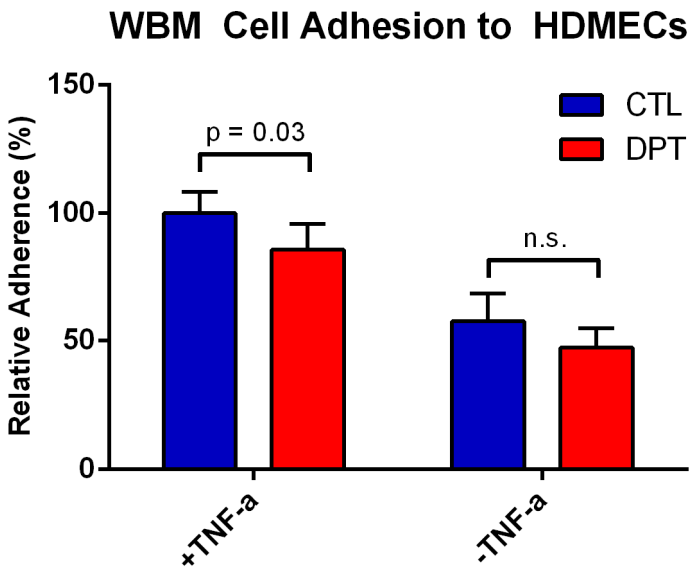

Figure 9: Relative adherence of WBM cells plated on top of HDMEC monolayers in the presence or absence of DPT. The fluorescently-labeled WBM cells were lysed after incubation. TNF-α activation of HDMECs nearly doubled the number of WBM cells that adhered to endothelial cells. DPT significantly reduced WBM cell adhesion to TNF-α-activated endothelial cells. CTL: control, DPT: dermatopontin, HDMEC: human dermal microvascular endothelial cell, TNF-α: tumor necrosis factor-alpha, n=12, n.s. = not significant.

Dermatopontin Releases Adhered WBM Cells

After observing DPT can reduce WBM cell adhesion to endothelial monolayers, we wondered if adding DPT to already adhered WBM cells would cause them to release. We developed a novel release assay which measures whether or not fluorescently labeled WBM cells un-attach from the endothelial cells when DPT was added to the system, as was measured using a plate reader and the supernatant from each well. Indeed, we observed that incubating adhered WBM cells with DPT could cause a substantial number of cells to release from endothelial monolayers (**Figure 10**). It should be noted, however,

that this experiment has only been performed once so further analysis is needed to confirm this phenomenon.

Figure 10: Dermatopontin causes adhered WBM cells to release from endothelial cells

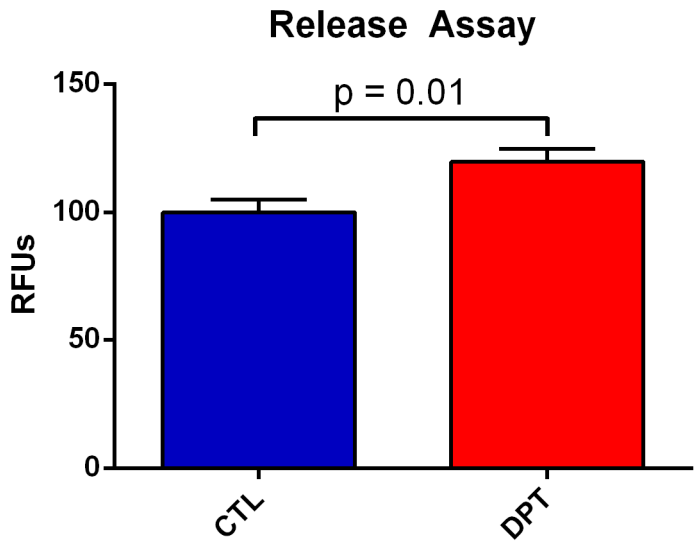

Figure 10: Release assay measuring whether or not DPT can cause already adhered WBM cells to release from endothelial cells into the media. After adhering to endothelial monolayers, fluorescently labeled WBM cells were incubated in the presence or absence of DPT. After incubation, the media was removed from the wells and any cells contained within were lysed. CTL: control, DPT: dermatopontin, RFUs: relative fluorescence units, n=10.

Dermatopontin Influences WBM Migration in vitro

Armed with the knowledge that DPT can reduce WBM cell adhesion to endothelial cells, we next examined if DPT affected WBM cell homing in transwell assays (**Figure 11**). In this assay, transwell baskets were first coated with fibronectin and then a monolayer of HUVECs. Cells did not migrate through the transwells unless SDF-1 was present in the bottom chamber, as expected, and DPT did not affect migration on

its own. The number of WBM cells that migrated through the transwell was significantly reduced when fibronectin was pre-coated on to the transwells. DPT was able significantly increase the number of migrated cells in the presence of fibronectin. Based on this evidence, we hypothesized that fibronectin was tightly binding to integrin receptors on migrating WBM cells and preventing further migration, trapping the cells at the transwell interface. We further hypothesized that when DPT was added to the system it interfered with these integrin receptors and inhibited their engagement with fibronectin, allowing the migrating WBM cells to more easily pass through the fibronectin coated transwells. We are currently investigating this phenomenon further.

Figure 11: Dermatopontin affects WBM cell migration in transwell migration assays

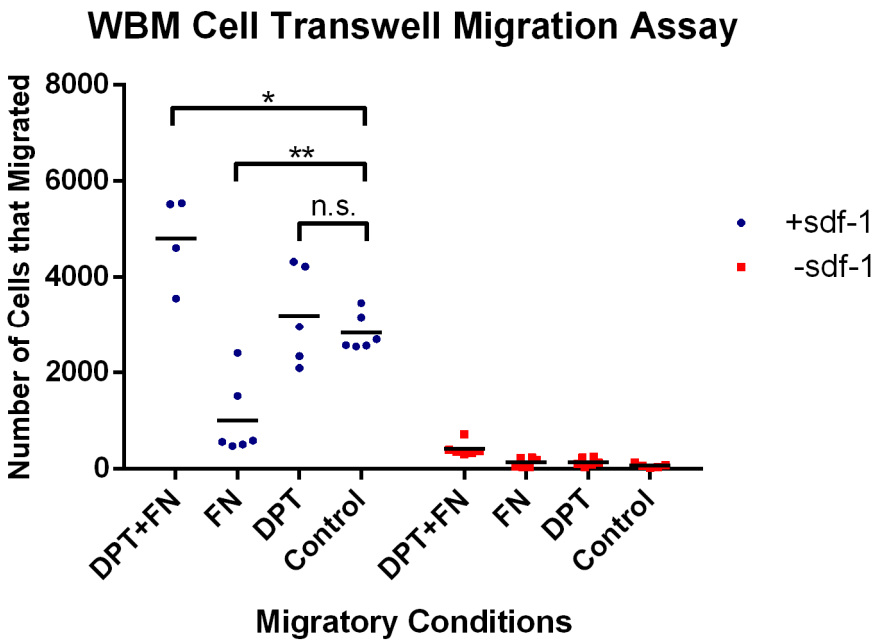

Figure 11: Transwell migration assaying showing the effect of DPT on WBM cell homing through a FN coated transwell covered in a monolayer of endothelial cells. Transwell baskets were coated with FN prior to seeding with HUVECs. Control wells were not coated with FN, but had a HUVEC monolayer. After HUVECs formed a confluent monolayer in the basket, CT Green-stained WBM cells were migrated against a gradient of SDF-1. Control migrations without sdf-1 are also represented. 1 µg of DPT was mixed with the cells prior to inoculation into the transwell basket. The number of cells which migrated into the bottom chamber was counted the next day using a flow cytometer. DPT: dermatopontin, FN: fibronectin, HUVECs: human umbilical vein endothelial cells, SDF-1: stromal derived factor-1, n=10.

Identification of Dermatopontin-Binding Integrins

We hypothesized that DPT may be interacting with integrin receptors and possibly blocking their function. Because the integrin VLA-5 is important in cell adhesion and homing, we chose to focus our efforts on characterizing its interaction with DPT. We also focused on a relatively understudied integrin, VLA-3, because it was reported in the literature to interact with DPT[74]. We first wanted to confirm that VLA-3

and VLA-5 play a role in cellular adherence, so we incubated BMECs with functional blocking antibodies against VLA-3 (anti-integrin α3 + anti-integrin β1 antibodies) and VLA-5 (anti-integrin α5 + anti-integrin β1 antibodies) and saw a marked decrease in cell adhesion to plastic (**Figure 12**), confirming the role of these integrins in cell adhesion to plastic.

Figure 12: Integrin blocking antibodies reduce BMEC adhesion

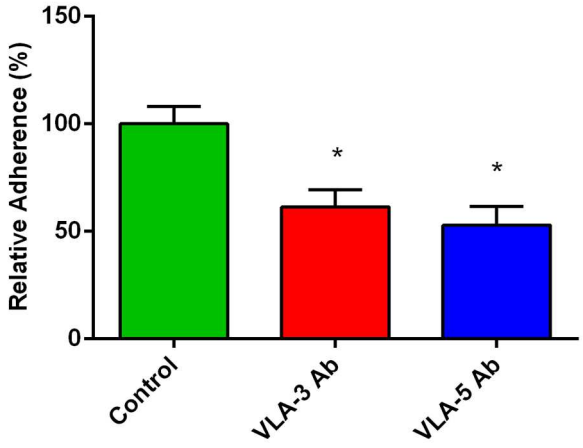

Figure 12: Relative adherence of BMECs incubated with integrin blocking antibodies prior to plating. The fluorescently-labeled WBM cells were lysed after incubation. Blocking both integrins significantly decreased cell adhesion compared to the control. * $P < 0.01$, n=6. BMEC: bone marrow endothelial cell, CTL: control, VLA: very-late antigen.

To determine if DPT was directly interacting with the integrin receptors VLA-3 and -5 on endothelial cells, we first incubated HUVECs with functional blocking antibodies against the α3, α5, and β1 subunits followed by incubation with DPT-488. We expected the blocking antibodies would prevent the labeled DPT from efficiently binding to the HUVECs. However, we saw no observable difference between the blocking antibodies and control treatments (**Figure 13A**). It was thought that the antibodies for

each individual subunit were not enough to prevent DPT binding, so we instead blocked with an antibody combination consisting of α3 + β1 (VLA-3) or α5 + β1 (VLA-5). The combination of α3 and β1 antibodies significantly decreased the number of HUVECs binding DPT-488 (**Figure 13B**). These findings suggest the integrin receptor VLA-3 is directly involved with dermatopontin binding to endothelial cells. This evidence also agrees with a previous report indicating VLA-3 interacts with DPT during keratinocyte adhesion. It should be noted that these data were obtained from a single experiment that has not been repeated yet.

Figure 13: Antibodies against VLA-3 prevent HUVECs from binding DPT

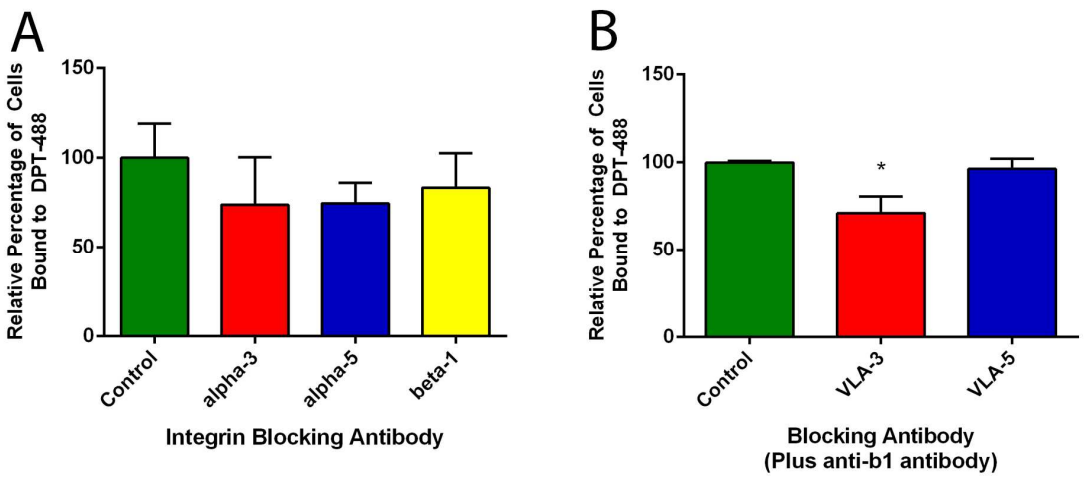

Figure 13: Relative percentage of cells bound to DPT-488 after incubation with individual (A) or a mixture (B) of integrin blocking antibodies. (A) Functional blocking antibodies against integrins α3, α5, and β1 were incubated with HUVECs prior to addition of DPT-488. These antibodies on their own did not significantly reduce DPT-488 binding to HUVECs. (B) Functional blocking antibodies against α3 + β1 (VLA-3) or α5 + β1 (VLA-5) were incubated with HUVECs prior to addition of DPT-488. We found that blocking VLA-3 reduced the number of cells binding to labeled DPT. * $P < 0.01$, n=3. DPT-488: Alexa Fluor® 488-labeled dermatopontin, HUVEC: human umbilical vein endothelial cell, VLA: very-late antigen.

Discussion

Unlike other non-collagenous proteins, dermatopontin has not been extensively studied despite being a widely distributed throughout the body. After it was discovered, experiments showed that DPT interacts with a number of other ECM molecules including collagen and fibronectin. However, the exact physiological function of DPT has not been described. These former studies primarily investigated DPT in the context of the skin and there have been no reports on the role of DPT in the hematopoietic system or bone marrow. Therefore, the goals of this study were to 1) characterize how DPT affects adhesion of hematopoietic and endothelial cells *in vitro* and 2) determine a possible mechanism by which DPT can negatively regulate HSC homing.

As previously described, our lab found that DPT negatively regulates HSC homing in mice. Mice treated with DPT prior to HCT show poor homing and engraftment compared to their untreated counterparts, which has been demonstrated in numerous transplant experiments. We found that DPT is secreted in the bone marrow by resident adipocytes, especially after treatment with radiation. Previous studies have hypothesized that DPT may be involved in wound healing processes in the skin and that it interacts with other ECM proteins to modify cell adhesion[71,78]. Similarly, we hypothesized that adipocytes highly express DPT in response to the massive injury caused by radiation and chemotherapy. We believe this secreted DPT then hinders successful HSC homing and engraftment, likely by modification of cell adhesion via altering the ECM or integrin receptors.

Contrary to previous reports that showed DPT increased cell adhesion[74,78], we consistently found that DPT decreased the overall cell adhesion of WBM cells and various types of endothelial cells. These results may indicate that DPT has a quite dynamic effect on cell adhesion. This effect may depend on the cell type and the presence of addition ECM molecules. In agreement with this, we found that in the case of HUVEC adhesion, DPT would not decrease cell adhesion unless in the presence of vitronectin.

Interestingly, we found that DPT reduces WBM cell adhesion to endothelial cells. We also showed that DPT could release WBM cells that were already adhered to endothelial cells. These results hint at a possible mechanism by which DPT can negatively regulate homing. DPT secreted by adipocytes and other cells into the bone marrow may be "coating" the vasculature and preventing transplanted HSCs from efficiently adhering to the endothelial cells. Confirming this, we demonstrated that DPT readily coats the lumen of intact aorta. As an alternative mechanism, DPT secreted into the blood may also be binding the circulating HSCs and preventing them from undergoing necessary tight adhesion to endothelial cells. In the case of either mechanism the HSCs would not be able to reach the hematopoietic niche since they would be trapped in circulation. This has been preliminarily confirmed by CFU assays, which showed an increased number of CFUs in the peripheral blood of DPT-treated animals compared to controls. Altogether, these data may indicate that DPT has a physiological function that negatively regulates the homing process during endogenous HSC mobilization.

However, the exact mechanism by which DPT impedes homing remains unclear. A number of previous papers have demonstrated the importance of integrin receptors binding DPT[73,74,79]. We believe that DPT lining the lumen of the bone marrow vasculature or coating circulating WBM cells may be inhibiting critical integrin receptors, likely VLA-3 and -5, thus preventing stable adhesion of the HSC to the endothelial cells. In this study we were able to allude to the involvement of VLA-3 in binding to labeled DPT. To further test this hypothesis we are now conducting pull-down assays to look if VLA-3 and -5 directly bind and interact with DPT. VLA-3 knockout mice and cell lines will also be a useful tool in validating our findings.

We are also in active development of a DPT knockout mouse to further aid in our investigation of DPT (unfortunately, colonies of the previously developed knockout mouse were completely destroyed). If our hypotheses are correct, DPT knockout mice should engraft substantially better due to a lack of DPT in their system. This will be the first documentation of a secreted adipokine able to impede hematopoietic cell homing.

References

1. Thomas ED, Lochte HL, Lu WC, Ferrebee JW. Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. *N Engl J Med*. 1957;257(11):491-496. doi:10.1056/NEJM195709122571102.

2. Thomas E, Storb R, Clift R a, et al. Bone-marrow transplantation. *N Engl J Med*. 1975;292(16):832-843. doi:10.1056/NEJM197504172921605.

3. Thomas ED, Lochte HL, Cannon JH, Sahler OD, Ferrebee JW. Supralethal whole body irradiation and isologous marrow transplantation in man. *J Clin Invest*. 1959;38:1709-1716. doi:10.1172/JCI103949.

4. Jacobson, LO, Marks, EK, Robson, MJ, Gaston, EO and Zirkle R. Effect of spleen protection on mortality following X-irradiation. *J Lab Clin Med*. 1949;34:1538-1543. http://www.osti.gov/scitech/biblio/4449544.

5. Jacobson LO, Simmons EL, Marks EK, Eldredge JH. Recovery from radiation injury. *Science*. 1951;113(2940):510-511. doi:10.1126/science.113.2940.510.

6. Cole LJ, Fishler MC, Ellis ME, Bond VP. Protection of Mice Against X-Irradiation by Spleen Homogenates Administered After Exposure. *Exp Biol Med*. 1952;80(1):112-117. doi:10.3181/00379727-80-19540.

7. Vos O, Davids J a, Weyzen WW, Van Bekkum DW. Evidence for the cellular hypothesis in radiation protection by bone marrow cells. *Acta Physiol Pharmacol Neerl*. 1956;4(4):482-486. http://www.ncbi.nlm.nih.gov/pubmed/13313162.

8. Nowell PC, Cole LJ, Habermeyer JG, Roan PL. Growth and continued function of rat marrow cells in x-radiated mice. *Cancer Res*. 1956;16(3):258-261. http://www.ncbi.nlm.nih.gov/pubmed/13304871.

9. Becker AJ, Mcculloch EA, Till JE. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature*. 1963;197:452-454. doi:10.1038/197452a0.

10. Siminovitch L, Mcculloch EA, Till JE. The Distribution of Colony-Forming Cells Among Spleen Colonies. *J Cell Physiol*. 1963;62(3):327-336. doi:10.1002/jcp.1030620313.

11. Copelan EA. Hematopoietic Stem-Cell Transplantation. *N Engl J Med*. 2006;354(17):1813-1826. http://www.nejm.org/doi/full/10.1056/nejmra052638.

12. Gatti R a, Meuwissen HJ, Allen HD, Hong R, Good R a. Immunological reconstitution of sex-linked lymphopenic immunological deficiency. *Lancet*. 1968;2(7583):1366-1369. doi:10.1016/S0140-6736(68)92673-1.

13. Appelbaum F. Hematopoietic Stem-Cell Transplantation at 50. *N Engl J Med*. 2007;357(15):1472-1475. http://www.nejm.org/doi/full/10.1056/nejmra052638.

14. Gratwohl A, Baldomero H, Aljurf M, et al. Hematopoietic stem cell transplantation: A global perspective. *JAMA*. 2011;303(16):1617-1624. doi:10.1001/jama.2010.491.Hematopoietic.

15. Passweg JR, Baldomero H, Gratwohl a, et al. The EBMT activity survey: 1990–2010. *Bone Marrow Transplant*. 2012;47(7):906-923. doi:10.1038/bmt.2012.66.

16. Hamilton BK, Copelan E a. Concise review: The role of hematopoietic stem cell transplantation in the treatment of acute myeloid leukemia. *Stem Cells*. 2012;30(8):1581-1586. doi:10.1002/stem.1140.

17. Peters C, Steward CG. Hematopoietic cell transplantation for inherited metabolic diseases: an overview of outcomes and practice guidelines. *Bone Marrow Transplant*. 2003;31(4):229-239. doi:10.1038/sj.bmt.1703839.

18. Coggle JE, Gordon MY. Quantitative measurements on the haemopoietic systems of three strains of mice. *Exp Hematol*. 1975;3(3):181-186. http://europepmc.org/abstract/med/1098920.

19. Abkowitz JL, Catlin SN, McCallie MT, Guttorp P. Evidence that the number of hematopoietic stem cells per animal is conserved in mammals. *Blood*. 2002;100(7):2665-2667. doi:10.1182/blood-2002-03-0822.

20. Spangrude GJ. Hematopoietic stem-cell differentiation. *Curr Opin Immunol*. 1991;3(2):171-178. doi:10.1016/0952-7915(91)90046-4.

21. Akala OO, Clarke MF. Hematopoietic stem cell self-renewal. *Curr Opin Genet Dev*. 2006;16(5):496-501. doi:10.1016/j.gde.2006.08.011.

22. Calvi LM, Adams GB, Weibrecht KW, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. *October*. 2003. doi:10.1038/nature02041.1.

23. Kunisaki Y, Bruns I, Scheiermann C, et al. Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature*. 2013;502(7473):637-643. doi:10.1038/nature12612.

24. Arai F, Suda T. Maintenance of quiescent hematopoietic stem cells in the osteoblastic niche. *Ann N Y Acad Sci*. 2007;1106:41-53. doi:10.1196/annals.1392.005.

25. Arai F, Hirao A, Ohmura M, et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell*. 2004;118(2):149-161. doi:10.1016/j.cell.2004.07.004.

26. Moore KA, Lemischka IR. "Tie-ing" down the hematopoietic niche. *Cell*. 2004;118(2):139-140. doi:10.1016/j.cell.2004.07.006.

27. Moore KA, Lemischka IR. Stem cells and their niches. *Science*. 2006;311(5769):1880-1885. doi:10.1126/science.1110542.

28. Williams WJ, Nelson DA. Examination of the marrow. *Hematol New York McGraw-Hill*. 1995:15-22.

29. Bianco P. Bone and the hematopoietic niche: a tale of two stem cells. *Blood*. 2011;117(20):5281-5288. doi:10.1182/blood-2011-01-315069.

30. Adams GB, Chabner KT, Alley IR, et al. Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. *Nature*. 2006;439(7076):599-603. doi:10.1038/nature04247.

31. Stier S, Ko Y, Forkert R, et al. Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. *J Exp Med*. 2005;201(11):1781-1791. doi:10.1084/jem.20041992.

32. Nagasawa T, Omatsu Y, Sugiyama T. Control of hematopoietic stem cells by the bone marrow stromal niche: the role of reticular cells. *Trends Immunol*. 2011;32(7):315-320. doi:10.1016/j.it.2011.03.009.

33. Lapidot T, Dar A, Kollet O. How do stem cells find their way home? *Blood*. 2005;106(6):1901-1910. doi:10.1182/blood-2005-04-1417.

34. Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity*. 2006;25(6):977-988. doi:10.1016/j.immuni.2006.10.016.

35. Peled A, Kollet O, Ponomaryov T, et al. The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. *Blood*. 2000;95(11):3289-3296. http://www.bloodjournal.org/content/95/11/3289.abstract.

36. Méndez-Ferrer S, Lucas D, Battista M, Frenette PS. Haematopoietic stem cell release is regulated by circadian oscillations. *Nature*. 2008. doi:10.1038/nature06685.

37. Goodman JW, Hodgson GS. Evidence for stem cells in the peripheral blood of mice. *Blood*. 1962;19(6):702-714. http://www.bloodjournal.org/content/19/6/702.abstract.

38. Richman CM, Weiner RS, Yankee RA. Increase in circulating stem cells following chemotherapy in man. *Blood*. 1976;47(6):1031-1039. http://www.ncbi.nlm.nih.gov/pubmed/1276467.

39. Lorenz E, Congdon C, Uphoff D. Modification of acute irradiation injury in mice and guinea-pigs by bone marrow injections. *Radiology*. 1952;58(6):863-877. doi:10.1148/58.6.863.

40. Kavanagh DPJ, Kalia N. Hematopoietic stem cell homing to injured tissues. *Stem Cell Rev*. 2011;7(3):672-682. doi:10.1007/s12015-011-9240-z.

41. Kollet O, Shivtiel S, Chen Y-Q, et al. HGF, SDF-1, and MMP-9 are involved in stress-induced human CD34+ stem cell recruitment to the liver. *J Clin Invest*. 2003;112(2):160-169. doi:10.1172/JCI17902.

42. Nilsson SK, Simmons PJ. Transplantable stem cells: home to specific niches. *Curr Opin Hematol*. 2004;11(2):102-106. doi:10.1097/01.moh.0000133651.06863.9c.

43. Papayannopoulou T. Bone marrow homing: the players, the playfield, and their evolving roles. *Curr Opin Hematol*. 2003;10(3):214-219. doi:10.1097/00062752-200305000-00004.

44. Aiuti a, Webb IJ, Bleul C, Springer T, Gutierrez-Ramos JC. The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. *J Exp Med*. 1997;185(1):111-120. doi:10.1084/jem.185.1.111.

45. Van Overstraeten-Schlögel N, Beguin Y, Gothot A. Role of stromal-derived factor-1 in the hematopoietic-supporting activity of human mesenchymal stem cells. *Eur J Haematol*. 2006;76(6):488-493. doi:10.1111/j.1600-0609.2006.00633.x.

46. Ponomaryov T, Peled A, Petit I, et al. Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function. *J Clin Invest*. 2000;106(11):1331-1339. doi:10.1172/JCI10329.

47. Ceradini DJ, Kulkarni AR, Callaghan MJ, et al. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nat Med*. 2004;10(8):858-864. doi:10.1038/nm1075.

48. Hidalgo A, Weiss L a., Frenette PS. Functional selectin ligands mediating human CD34+ cell interactions with bone marrow endothelium are enhanced postnatally. *J Clin Invest*. 2002;110(4):559-569. doi:10.1172/JCI200214047.

49. Mazo IB, Gutierrez-Ramos JC, Frenette PS, Hynes RO, Wagner DD, von Andrian UH. Hematopoietic progenitor cell rolling in bone marrow microvessels: parallel contributions by endothelial selectins and vascular cell adhesion molecule 1. *J Exp Med*. 1998;188(3):465-474. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2212463&tool=pmcentrez&rendertype=abstract.

50. Hidalgo A, Sanz-Rodríguez F, Rodríguez-Fernández JL, et al. Chemokine stromal cell-derived factor-1alpha modulates VLA-4 integrin-dependent adhesion to fibronectin and VCAM-1 on bone marrow hematopoietic progenitor cells. *Exp Hematol*. 2001;29(3):345-355. http://www.ncbi.nlm.nih.gov/pubmed/11274763.

51. Driessen RL, Johnston HM, Nilsson SK. Membrane-bound stem cell factor is a key regulator in the initial lodgment of stem cells within the endosteal marrow region. *Exp Hematol*. 2003;31(12):1284-1291. http://www.ncbi.nlm.nih.gov/pubmed/14662336.

52. Nilsson SK, Haylock DN, Johnston HM, Occhiodoro T, Brown TJ, Simmons PJ. Hyaluronan is synthesized by primitive hemopoietic cells, participates in their lodgment at the endosteum following transplantation, and is involved in the regulation of their proliferation and differentiation in vitro. *Blood*. 2003;101(3):856-862. doi:10.1182/blood-2002-05-1344.

53. Nilsson SK, Johnston HM, Whitty GA, et al. Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. *Blood*. 2005;106(4):1232-1239. doi:10.1182/blood-2004-11-4422.

54. Heazlewood SY, Oteiza A, Cao H, Nilsson SK. Analyzing hematopoietic stem cell homing, lodgment, and engraftment to better understand the bone marrow niche. *Ann N Y Acad Sci*. 2014;1310:119-128. doi:10.1111/nyas.12329.

55. Lanzkron SM, Collector MI, Sharkis SJ. Hematopoietic stem cell tracking in vivo: a comparison of short-term and long-term repopulating cells. *Blood*. 1999;93(6):1916-1921. http://www.bloodjournal.org/content/93/6/1916.abstract.

56. Mattsson J, Ringdén O, Storb R. Graft Failure after Allogeneic Hematopoietic Cell Transplantation. *Biol Blood Marrow Transplant*. 2008;14(Supplement 1):165-170. doi:10.1016/j.bbmt.2007.10.025.

57. Olsson R, Remberger M, Schaffer M, et al. Graft failure in the modern era of allogeneic hematopoietic SCT. *Bone Marrow Transplant*. 2013;48(4):537-543. doi:10.1038/bmt.2012.239.

58. Davies SM, Kollman C, Anasetti C, et al. Engraftment and survival after unrelated-donor bone marrow transplantation: a report from the national marrow donor program. *Blood*. 2000;96(13):4096-4102. http://www.ncbi.nlm.nih.gov/pubmed/11110679.

59. Remberger M, Mattsson J, Olsson R, Ringdén O. Second allogeneic hematopoietic stem cell transplantation: A treatment for graft failure. *Clin Transplant*. 2011;25(1):E68-E76. doi:10.1111/j.1399-0012.2010.01324.x.

60. Schriber J, Agovi M-A, Ho V, et al. Second unrelated donor hematopoietic cell transplantation for primary graft failure. *Biol Blood Marrow Transplant*. 2010;16(8):1099-1106. doi:10.1016/j.bbmt.2010.02.013.

61. Quinones RR. Hematopoietic engraftment and graft failure after bone marrow transplantation. *Am J Pediatr Hematol Oncol*. 1993;15(1):3-17. doi:10.1097/00043426-199302000-00002.

62. Giriş M, Erbil Y, Oztezcan S, et al. The effect of heme oxygenase-1 induction by glutamine on radiation-induced intestinal damage: the effect of heme oxygenase-1 on radiation enteritis. *Am J Surg*. 2006;191(4):503-509. doi:10.1016/j.amjsurg.2005.11.004.

63. Yokota T, Oritani K, Takahashi I, et al. Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood*. 2000;96(5):1723-1732. http://www.bloodjournal.org/content/96/5/1723.abstract.

64. Sugiyama T, Nagasawa T. Bone marrow niches for hematopoietic stem cells and immune cells. *Inflamm Allergy Drug Targets*. 2012;11(3):201-206. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3405285&tool=pmcentrez&rendertype=abstract.

65. Anthony BA, Link DC. Regulation of hematopoietic stem cells by bone marrow stromal cells. *Trends Immunol*. 2014;35(1):32-37. doi:10.1016/j.it.2013.10.002.

66. Naveiras O, Nardi V, Wenzel PL, Hauschka P V, Fahey F, Daley GQ. Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. *Nature*. 2009;460(7252):259-263. doi:10.1038/nature08099.

67. Belaid-Choucair Z, Lepelletier Y, Poncin G, et al. Human bone marrow adipocytes block granulopoiesis through neuropilin-1-induced granulocyte colony-stimulating factor inhibition. *Stem Cells*. 2008;26(6):1556-1564. doi:10.1634/stemcells.2008-0068.

68. Miharada K, Hiroyama T, Sudo K, Danjo I, Nagasawa T, Nakamura Y. Lipocalin 2-mediated growth suppression is evident in human erythroid and monocyte/macrophage lineage cells. *J Cell Physiol*. 2008;215(2):526-537. doi:10.1002/jcp.21334.

69. Zhang Y, Harada a, Bluethmann H, et al. Tumor necrosis factor (TNF) is a physiologic regulator of hematopoietic progenitor cells: increase of early hematopoietic progenitor cells in TNF receptor p55-deficient mice in vivo and potent inhibition of progenitor cell proliferation by TNF alpha in vi. *Blood*. 1995;86(8):2930-2937.

70. Neame P, Choi H, Rosenberg L. The isolation and primary structure of a 22-kDa extracellular matrix protein from bovine skin. *J Biol Chem*. 1989;264(10):5474-5479.

71. Okamoto O, Fujiwara S. Dermatopontin, a novel player in the biology of the extracellular matrix. *Connect Tissue Res*. 2006;47(4):177-189. doi:10.1080/03008200600846564.

72. Wilmarth KR, Froines JR. In vitro and in vivo inhibition of lysyl oxidase by aminopropionitriles. *J Toxicol Environ Health*. 1992;37(3):411-423. doi:10.1080/15287399209531680.

73. Ruoslahti E. RGD and other recognition sequences for integrins. *Annu Rev Cell Dev Biol*. 1996;12:697-715. doi:10.1146/annurev.cellbio.12.1.697.

74. Okamoto O, Hozumi K, Katagiri F, et al. Dermatopontin promotes epidermal keratinocyte adhesion via alpha3beta1 integrin and a proteoglycan receptor. *Biochemistry*. 2010;49(1):147-155. doi:10.1021/bi901066f.

75. Liu X, Meng L, Shi Q, et al. Dermatopontin promotes adhesion, spreading and migration of cardiac fibroblasts in vitro. *Matrix Biol*. 2013;32(1):23-31. doi:10.1016/j.matbio.2012.11.014.

76. Kato A, Okamoto O, Wu W, et al. Identification of fibronectin binding sites in dermatopontin and their biological function. *J Dermatol Sci*. 2014;76(1):51-59. doi:10.1016/j.jdermsci.2014.07.003.

77. Wu W, Okamoto O, Kato A, et al. Functional peptide of dermatopontin produces fibrinogen fibrils and modifies its biological activity. *J Dermatol Sci*. 2014;76(1):34-43. doi:10.1016/j.jdermsci.2014.07.002.

78. Kato A, Okamoto O, Ishikawa K, et al. Dermatopontin interacts with fibronectin, promotes fibronectin fibril formation, and enhances cell adhesion. *J Biol Chem*. 2011;286(17):14861-14869. doi:10.1074/jbc.M110.179762.

79. Wu W, Okamoto O, Kato A, et al. Dermatopontin regulates fibrin formation and its biological activity. *J Invest Dermatol*. 2014;134(1):256-263. doi:10.1038/jid.2013.305.

80. Yamatoji M, Kasamatsu A, Kouzu Y, et al. Dermatopontin: a potential predictor for metastasis of human oral cancer. *Int J Cancer*. 2012;130(12):2903-2911. doi:10.1002/ijc.26328.

81. Takeuchi T. Structual comparison of dermatopontin amino acid sequences. *Biologia (Bratisl)*. 2010;65(5):874-879. doi:10.2478/s11756-010-0098-3.

82. Superti-Furga A, Rocchi M, Schäfer BW, Gitzelmann R. Complementary DNA sequence and chromosomal mapping of a human proteoglycan-binding cell-adhesion protein (dermatopontin). *Genomics*. 1993;17(2):463-467. doi:10.1006/geno.1993.1348.

83. Tan Y, Iimura K, Sato T, Ura K, Takagi Y. Spatiotemporal expression of the dermatopontin gene in zebrafish Danio rerio. *Gene*. 2013;516(2):277-284. doi:10.1016/j.gene.2012.11.074.

84. Sarashina I, Yamaguchi H, Haga T, Iijima M, Chiba S, Endo K. Molecular evolution and functionally important structures of molluscan Dermatopontin: implications for the origins of molluscan shell matrix proteins. *J Mol Evol*. 2006;62(3):307-318. doi:10.1007/s00239-005-0095-2.

85. Forbes EG, Cronshaw AD, MacBeath JRE, Hulmes DJS. Tyrosine-rich acidic matrix protein (TRAMP) is a tyrosine-sulphated and widely distributed protein of the extracellular matrix. *FEBS Lett*. 1994;351(3):433-436. doi:10.1016/0014-5793(94)00907-4.

86. Cooper LJ, Bentley AJ, Nieduszynski IA, et al. The role of dermatopontin in the stromal organization of the cornea. *Invest Ophthalmol Vis Sci*. 2006;47(8):3303-3310. doi:10.1167/iovs.05-1426.

87. Li X, Feng P, Ou J, et al. Dermatopontin is expressed in human liver and is downregulated in hepatocellular carcinoma. *Biochem*. 2009;74(9):979-985. doi:10.1134/S0006297909090053.

88. Behnam K, Murray SS, Brochmann EJ. BMP stimulation of alkaline phosphatase activity in pluripotent mouse C2C12 cells is inhibited by dermatopontin, one of the most abundant low molecular weight proteins in demineralized bone matrix. *Connect Tissue Res*. 2006;47(5):271-277. doi:10.1080/03008200600995908.

89. Okamoto O, Fujiwara S, Abe M, Sato Y. Dermatopontin interacts with transforming growth factor beta and enhances its biological activity. *Biochem J*. 1999;337 (Pt 3:537-541. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1220007&tool=pmcentrez&rendertype=abstract.

90. Takeda U, Utani A, Wu J, et al. Targeted disruption of dermatopontin causes abnormal collagen fibrillogenesis. *J Invest Dermatol*. 2002;119(3):678-683. doi:10.1046/j.1523-1747.2002.01863.x.

91. Cooper LJ, Bentley AJ, Nieduszynski IA, et al. An ultrastructural investigation of dermatopontin-knockout mouse corneas. *Int J Exp Pathol*. 2004;85(4):A59-A60. doi:10.1111/j.0959-9673.2004.0390r.x.

92. Yang Q, Hao J, Chen M, Li G. Dermatopontin is a novel regulator of the CdCl2-induced decrease in claudin-11 expression. *Toxicol In Vitro*. 2014;28(6):1158-1164. doi:10.1016/j.tiv.2014.05.013.

93. Takemoto S, Murakami T, Kusachi S, et al. Increased expression of dermatopontin mRNA in the infarct zone of experimentally induced myocardial infarction in rats: comparison with decorin and type I collagen mRNAs. *Basic Res Cardiol*. 2002;97(6):461-468. doi:10.1007/s00395-002-0371-x.

94. Le Blanc K, Ringdén O. Mesenchymal stem cells: properties and role in clinical bone marrow transplantation. *Curr Opin Immunol*. 2006;18(5):586-591. doi:10.1016/j.coi.2006.07.004.

95. Devine SM, Hoffman R. Role of mesenchymal stem cells in hematopoietic stem cell transplantation. *Curr Opin Hematol*. 2000;7(6):358-363. http://ovidsp.tx.ovid.com.ezp1.lib.umn.edu/sp-3.15.1b/ovidweb.cgi?QS2=434f4e1a73d37e8c87ee6dca549dcede5ecc05f2149989e

a488e34142d428051ec4f45b133d80de026653f164ed6178601110f891567ab7dfee6
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e58316323319f12b2dfe71e453ee8d0338e4df9b605cad01d6165015b4c0b57c46afd
53e639034bdbe65f77f65fca7e380a764f3868. Accessed May 28, 2015.

96. Paffett-Lugassy NN, Zon LI. Analysis of hematopoietic development in the zebrafish. In: *Methods in Molecular Medicine*.Vol 105. Springer; 2005:171-198. doi:10.1385/1-59259-826-9:171.

97. Brizzi MF, Tarone G, Defilippi P. Extracellular matrix, integrins, and growth factors as tailors of the stem cell niche. *Curr Opin Cell Biol*. 2012;24(5):645-651. doi:10.1016/j.ceb.2012.07.001.

98. Kimizuku F, Taguchi Y, Ohdate Y, et al. Production and Characterization of Functional Domains of Human Fibronectin Expressed in Escherichia coli. *J Biochem*. 1991;110(2):284-291. http://jb.oxfordjournals.org.ezp2.lib.umn.edu/content/110/2/284.short.

99. Fukui N, Fukuda A, Kojima K, Nakajima K, Oda H, Nakamura K. Suppression of fibrous adhesion by proteoglycan decorin. *J Orthop Res*. 2001;19(3):456-462. doi:10.1016/S0736-0266(00)90016-0.

100. Winnemöller M, Schmidt G, Kresse H. Influence of decorin on fibroblast adhesion to fibronectin. *Eur J Cell Biol*. 1991;54(1):10-17. http://europepmc.org/abstract/med/1827765.

101. Zhang F, Cheng J, Lam G, et al. Adenovirus vector E4 gene regulates connexin 40 and 43 expression in endothelial cells via PKA and PI3K signal pathways. *Circ Res*. 2005;96(9):950-957. doi:10.1161/01.RES.0000165867.95291.7b.

102. Seandel M, Butler JM, Kobayashi H, et al. Generation of a functional and durable vascular niche by the adenoviral E4ORF1 gene. *Proc Natl Acad Sci U S A*. 2008;105(49):19288-19293. doi:10.1073/pnas.0805980105.

103. Gotsch U, Jäger U, Dominis M, Vestweber D. Expression of P-selectin on endothelial cells is upregulated by LPS and TNF-alpha in vivo. *Cell Adhes Commun*. 1994;2(1):7-14. http://www.ncbi.nlm.nih.gov/pubmed/7526954.

104. Wyble CW, Hynes KL, Kuchibhotla J, Marcus BC, Hallahan D, Gewertz BL. TNF-alpha and IL-1 upregulate membrane-bound and soluble E-selectin through a common pathway. *J Surg Res*. 1997;73(2):107-112. doi:10.1006/jsre.1997.5207.

105. Mackay F, Loetscher H, Stueber D, Gehr G, Lesslauer W. Tumor necrosis factor alpha (TNF-alpha)-induced cell adhesion to human endothelial cells is under dominant control of one TNF receptor type, TNF-R55. *J Exp Med*. 1993;177(5):1277-1286. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2190994&tool=pmcentrez&rendertype=abstract.