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I, Megan Pirman, hereby submit this original work as part of the requirements for the degree of:

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It is entitled:

An In Vitro Study on the Role of Endothelial Cell Connexin43 Gap Junctions in the Regulation of Hematopoietic Stem and Progenitor Cells Traffic

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An In Vitro Study on the Role of Endothelial Cell Connexin43 Gap Junctions in the Regulation of Hematopoietic Stem and Progenitor Cells Traffic

A thesis submitted to the Division of Research and Advanced Studies of the University of Cincinnati in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

In the Department of Transfusion and Transplantation Sciences of the College of Allied Health

Committee Chair: Jose A. Cancelas, MD, PhD

For the year 2009

By

Megan K. Pirman, MT (ASCP)CM

B.A. Thomas More College, Kentucky, 2005
ABSTRACT

Cell-to-cell contact between hematopoietic stem cells and progenitors (HSC/P) and their supporting bone marrow (BM) microenvironment has been shown to be pivotal in blood formation and hematopoietic homeostasis between BM and peripheral blood. BM derived endothelial cells (EC) form a major cell component in the hematopoietic microenvironment. BMEC are fenestrated to allow the traffic of hematopoietic cells to and from the circulation while maintaining adhesion and communication with other EC and HSC/P. Gap junctions (GJ) represent one system in which adjacent cells adhere in order to communicate intercellularly. GJ are channels constituted by a group of proteins called connexins (Cx). It is known that Cx43 GJ are involved in the interactions between hematopoietic cells and the BM microenvironment. Based on in vivo preliminary data indicating loss of retention of HSC/P in BM of endothelial-specific Cx43-deficient mice, we hypothesized that the loss of Cx43 would induce a decreased adhesion and/or increased migration of HSC/P, respectively, to or through Cx43-deficient EC.

Our results indicate that the deficiency of Cx43 in EC, induced by RNA interference induces decreased adhesion and increased transendothelial migration of normal HSC/P. However, the transendothelial migration of Cx43-deficient HSC/P through Cx43 RNA-silenced EC is not modified, suggesting that the mechanisms mediated by Cx43 involved in the control of transendothelial migration are more complex than anticipated. In summary, Cx43 deficiency in the EC compartment may induce a complex array of changes in the proliferation, adhesion and migration of HSC/P. Migration of HSC/P, however, seems to depend on the expression of Cx43 in both EC and HSC/P. Cx43 plays pleiotropic, cell-specific roles in the hematopoietic microenvironment.
ACKNOWLEDGMENTS

I would like to thank Dr. Cancelas and Dr. Kyung Hee Chang, for giving me a chance to work on such a challenging endeavor. I have learned an incredible amount in the tiny amount of time we shared. Dr. Cancelas, you have been a dedicated mentor to me. I truly appreciate you asking the challenging questions to help me grow, and taking the time in answering all my questions. Thank you for sharing your boundless wisdom with me, teaching me how to think like a scientist and of course for all your help with endless revisions of my thesis. Kyung Hee, thank you for all the time and experience you shared with me- including teaching me everything about endothelial cells and how to keep them “happy” in culture. To Daniel Gonzalez-Nieto for all your advice and teaching opportunities. You were my Cx43 and transendothelial migration assay guru 😊 To Amitava Sangupta, for answering any stray technical questions I had. Thank you Rebecca Santho for preparing (breeding, etc) the mice used in this project and teaching me how to “handle” them, and Susan Dunn for genotyping the mice and taking the time to explain the process to me.

I would also like to thank my mentor, advisor, and confidante, Pam Inglish, for all the guidance, encouragement and dedication you instilled in me. It has been quite a journey, thank you for always helping me stay on course. I would like to thank everyone else at Hoxworth, especially Margaret Simon, for making me feel as part of the team during rotations, and Margaret O’Leary, for all the technical advice on Microsoft word, power point and tips for presenting well (you helped make editing bearable). Naseem, it has been a pleasure knowing you, good luck. 😊 The learning experience I gained in these past 15 months I will take with me wherever I go. Finally, I would like to thank my husband, Ryan, for supporting me in every way imaginable and without whom I could never have finished this program! I love you. Thank you all again!

Best Wishes Always,

Megan
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>7-AAD</td>
<td>7-aminoactinomycin D</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BMEC</td>
<td>BM derived EC</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CAMs</td>
<td>cell adhesion molecules</td>
</tr>
<tr>
<td>CBC</td>
<td>complete blood counts</td>
</tr>
<tr>
<td>CCHMC</td>
<td>Cincinnati Children’s Hospital Medical Center</td>
</tr>
<tr>
<td>CD34</td>
<td>a cell-surface sialomucin, a hematopoietic and EC progenitor marker,</td>
</tr>
<tr>
<td>CD45</td>
<td>leukocyte common antigen</td>
</tr>
<tr>
<td>CD106</td>
<td>see VCAM-1</td>
</tr>
<tr>
<td>CD150</td>
<td>a marker for long-term hematopoietic stem cells</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CFU-C</td>
<td>colony-forming unit-content</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre recombinase</td>
</tr>
<tr>
<td>Cx</td>
<td>connexin(s)</td>
</tr>
<tr>
<td>CXCL12</td>
<td>see SDF-1α</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ECGS</td>
<td>EC growth supplement</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>g</td>
<td>gravity-force</td>
</tr>
<tr>
<td>GADPH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>G-CSF</td>
<td>granulocyte-colony stimulating factor</td>
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<tr>
<td>GJ</td>
<td>gap junctions</td>
</tr>
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<td>Gja1</td>
<td>GJ protein alpha 1 gene</td>
</tr>
<tr>
<td>GJIC</td>
<td>GJ intercellular channels</td>
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<tr>
<td>HSC</td>
<td>hematopoietic stem cells</td>
</tr>
<tr>
<td>HSC/P</td>
<td>hematopoietic stem and progenitor cells</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s modified Dulbecco’s medium</td>
</tr>
<tr>
<td>ITS</td>
<td>insulin, transferrin, selenium</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>KDR</td>
<td>kinase domain receptor, an EC growth factor receptor, also known as VEGFR-2</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LDBM</td>
<td>low density BM</td>
</tr>
<tr>
<td>LK</td>
<td>lineage-/c-kit+</td>
</tr>
<tr>
<td>mEPOC</td>
<td>murine endothelial progenitor cells</td>
</tr>
<tr>
<td>MFI</td>
<td>median fluorescent intensity</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
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</table>
MPP  multipotential hematopoietic progenitors (lineage-1/c-kit+/Sca-1+/CD34+/CD135+)
ODDD  oculodentodigital dysplasia
PB    peripheral blood
PBS   phosphate buffered saline
PCR   polymerase chain reaction
PE    phycoerythrin
PECAM platelet and endothelial cell adhesion molecule
PTH   parathyroid hormone
Q-PCR quantitative real time-PCR
Rb    retinoblastoma protein
RISC RNA-induced silencing complex
RNA   ribonucleic acid
RT-PCR reverse transcriptase-polymerase chain reaction
SCF   stem cell factor
SDF-1α stromal cell derived factor-1 alpha (also known as CXCL12)
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA short-hairpin RNA
shRNA A 3′ untranslated region on exon 2 of the Cx43 gene target
sequence (CCGGCCCACCTTTGTGTCTTCCATACTCGAGTAGTGGAAGACACAAAGGTGGGTTTTG)
shRNA E open translated region on exon 2 of the Cx43 gene target
sequence (CCGGGGCTCTTCTATGTCTTCTTCATACTCGAGTAGGAAGAAGACATAGAAGAGCTTTTTG)
shRNA/control non-target control sequence (CCGGCAACAAAGATGAAGAGCGAGGATAGAGAGCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
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<tr>
<td>SV40</td>
<td>simian virus large T antigen</td>
</tr>
<tr>
<td>TBSt</td>
<td>tris buffered saline working solution</td>
</tr>
<tr>
<td>Tek</td>
<td>angiopoietin-1 receptor, an EC marker, also known as Tie2</td>
</tr>
<tr>
<td>Tek-Cre KO</td>
<td>Tek-Cre;Cx43&lt;sup&gt;flox/flox&lt;/sup&gt; (transgenic KO)</td>
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<tr>
<td>Tek-Cre WT</td>
<td>Tek-Cre;Cx43 WT (transgenic WT)</td>
</tr>
<tr>
<td>Tie2</td>
<td>see Tek</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor – alpha</td>
</tr>
<tr>
<td>Tg/Ntg</td>
<td>transgenic/non-transgenic</td>
</tr>
<tr>
<td>Vav1</td>
<td>a hematopoietic promoter</td>
</tr>
<tr>
<td>Vav1-Cre KO</td>
<td>Vav1-Cre;Cx43&lt;sup&gt;flox/flox&lt;/sup&gt; (transgenic KO)</td>
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<tr>
<td>Vav1-Cre WT</td>
<td>Vav1-Cre;Cx43 WT (transgenic WT)</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1 (CD106) an adhesion molecule receptor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>vascular endothelial growth factor receptors</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>see KDR</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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INTRODUCTION

Hematopoietic stem cells (HSC) are the best-characterized adult stem cells in mammalian tissues. Since the identification of these self-renewing, blood-forming cells in the late 1960’s, technological advances have allowed for their purification and use in regenerative and transplantation medicine. For over 20 years, it has also been known that HSC are dependent on a specialized bone marrow (BM) microenvironment, called the HSC niche.\textsuperscript{1,2} However, the interrelationship between the HSC and their niche, and the anatomical, cellular and molecular basis of this niche remains unclear. Studies have shown that the BM microenvironment most likely consists of two anatomical niches, the vascular and endosteal niches, which may be highly interrelated due to their proximity. This microenvironment contains a network of supporting cells including stromal cells, osteoblasts, osteoclasts, reticular cells, and endothelial cells (EC), which produce intrinsic and extrinsic signals that communicate directly with one another and with HSC, to promote and maintain stem cell function, self-renewal, and differentiation, depending on their distribution and location in the niche.\textsuperscript{1,3,4} The mechanisms which regulate hematopoiesis via the stem cell niche are still only beginning to be elucidated. As we uncover these mechanisms, a greater understanding of the HSC niche(s) can lead to new therapeutic opportunities.

Three different mechanisms have been postulated to mediate the regulation of proliferation and differentiation of HSC by stromal cells: (a) by cytokine receptor-ligand interaction, (b) by interaction of adhesion molecules on hematopoietic cells with stromal cells or the extracellular matrix, or (c) by direct cell-to-cell communication between stromal cells or between stromal cells and hematopoietic
cells.\textsuperscript{5, 6} Among stromal cells, EC regulate the movement of HSC from the BM stem cell niches to circulation. We intend to study the relationship and interaction of EC in the BM vascular niche and hematopoietic stem and progenitor cells (HSC/P) through gap junctions (GJ). The existence of direct communication by GJ in BM has been known for over 25 years.\textsuperscript{7, 8} The predominant connexin (Cx) forming part of GJ in the BM microenvironment is Cx43.\textsuperscript{9, 10} In this work, we specifically intend to analyze the role of Cx43 in endothelium-dependent BM hematopoiesis.

**HSC Niches**

HSC/P require a unique microenvironment of growth factors, accessory cells and extracellular matrix proteins to maintain their quiescence and self-renewal ability. In the 1970’s, Schofield first recognized the relationship between HSC and bone, and postulated the existence of vaguely defined anatomically stem cell niches\textsuperscript{11} and an association between HSC location in the medullary cavity of the bone and the endosteal space was found.\textsuperscript{12} The endosteal niche resides near and along the surface of trabecular bone, which contains osteoblasts that directly communicate with HSC.\textsuperscript{1, 4} It is thought that the endosteal niche supports long-term, self-renewing HSC that are mostly quiescent in nature. In response to injury or stress, the endosteal osteoblasts and stromal cells release signals that activate the HSC to move toward the BM cavity and specifically toward the vascular niche, located near the endosteal niche.\textsuperscript{13}

The vascular niche was defined by Kiel et al in 2005. The vascular niche is composed of sinusoidal EC in close vicinity to HSC, phenotypically identified as CD150\textsuperscript{+} (a marker for long-term HSC). Sinusoids are specialized blood vessels lined with EC, which transport cells from BM into venous circulation. This group
discovered that two-thirds of HSC in the BM and spleen are located at these sinusoidal areas.\textsuperscript{14} It has also been suggested that HSC in contact with sinusoid EC would be short-term, self-renewing and actively dividing in nature, due to environmental stimulating factors in the vascular niche, and that CD150\textsuperscript{+} HSC in the area could constantly monitor these factors in order to meet the needs of the hematopoietic system.\textsuperscript{1,14,15}

In general, the hematopoietic niches have been described as micro-anatomical areas where restricted environmental cues are expressed, including “homing” ligands, usually cell adhesion molecules (CAMs) such as vascular cell adhesion molecule (VCAM-1, also known as CD106) or E-selectin, and chemokines such as stromal cell derived factor-1 alpha (SDF-1\textalpha, also known as CXCL12). These niches constitute a unique microenvironment for HSC/P homing and engraftment.\textsuperscript{16,17} The recruitment of incoming HSC from the endosteal niche is probably necessary to ensure prolonged HSC production and release from the vascular niche into the peripheral blood (PB) and/or exchange with other HSC pools.\textsuperscript{1} In fact, vascular structures throughout the BM appear to extend into most endosteal zones, indicating that the endosteal and vascular niche are not two separate microanatomical areas, but are interconnected and work together to regulate hematopoiesis.\textsuperscript{18}

**Bone Marrow Derived Endothelial Cells (BMEC)**

BMEC that line the sinusoids play an important role in HSC adhesion and/or migration (also called diapedesis) to and from the vascular niche. For this reason, BM vascular EC are often described as the “gatekeepers”. BMEC also support proliferation and differentiation of HSC/P via various hematopoietic growth factors
and cell-to-cell contact. Studies have shown that regeneration of BMEC in the vascular niche is essential for hematopoietic recovery after myeloablative therapy, such as radiation or 5-fluorouracil (5-FU) treatment. Such regeneration depends on vascular endothelial growth factor receptors (VEGFR)-1, VEGFR-2 (also known as KDR (kinase domain receptor)), and Tie2 (also known as angiopoietin receptor or Tek) expression, all of which are markers of EC. The resulting neoangiogenesis is necessary for engraftment of HSC/P and reconstitution of hematopoiesis.

The BMEC present in the vascular niche are functionally and phenotypically distinct from other, non-hematopoietic, vascular EC, which are found in other tissues. The BM vascular endothelium is fenestrated with large pores and gaps, and a discontinuous basal membrane, which allows the transfer of cells and plasma proteins. It also acts as a barrier for cell migration directly through physical connections, and chemically through the expression of specific chemorepellants and chemoattractants. A physiologically relevant chemoattractant expressed in large amounts by BMEC is SDF-1α, which is known to be involved in the trafficking of HSC/P. BMEC, like other EC, also express adhesion molecules such as E-selectin, platelet and endothelial cell adhesion molecule (PECAM) and VCAM-1 all of which are very important for HSC/P survival, proliferation, and differentiation.

The success of the engraftment in HSC transplantation depends on the migration of infused stem cells to the BM stroma, a process referred to as homing. Homing requires the transmigration of stem cells through the BM endothelium to settle in the BM niche, a process that is not yet fully understood. Adhesion of HSC/P to EC, their subsequent transmigration through the vascular endothelium, and retention of HSC/P within the BM cavity depends largely on expression of CAMs and
chemokines secreted by the hematopoietic microenvironment. Transendothelial migration and homing of transplanted HSC/P depends on the presence of a gradient of CXCL12 between the BM and the PB. CXCL12 is secreted in large amounts by BM stroma (including BMEC) and binds CXCR4, a seven-transmembrane domain chemokine receptor found on hematopoietic cells. CXCL12/CXCR4 signaling induces directional migration of HSC/P towards the BM microenvironment during homing. The adhesion and transendothelial migration assays are \textit{in vitro} surrogate tools used to study the homing mechanisms of HSC/P or other cells through EC and the adhesion molecules that are necessary for this process.

The expression of Cx43, a member of the GJ protein family, is another factor observed to be essential in the interaction of hematopoietic cells with EC. Veliz et al used a model to study the participation of Cx43 GJ in the leukocyte-endothelium interaction during an inflammatory response. They determined the adhesion of leukocytes to EC lead to their migration through the endothelium and that Cx43 expression is crucial to this process. They observed that leukocytes from Cx43 deficient mice did not adhere to the vascular endothelium when stimulated with tumor necrosis factor-alpha (TNF-α), a cytokine involved in the inflammatory response, compared to leukocytes from Cx43 wild type (WT) mice, suggesting impaired adhesion.

**Gap Junctions and Connexins**

GJ represent one well-known system of cell-to-cell communication and adhesion, which are important to many developmental and physiological processes. GJ intercellular channels (GJIC) are formed by Cx, individual structural protein units, which allow direct exchange of small molecules between the cytoplasm
of connected cells. GJIC exist in almost all mammalian cell types. GJ regulation of intercellular communication is achieved by highly complex processes involving junctional electric potentials, intracellular calcium levels, pH levels, Cx phosphorylation and other binding partners such as cadherins and tight junction proteins. Cx have also been shown to function independently of hemichannel formation and intercellular communication, being essential in the development and regulation of cell proliferation, resistance to injury, and cell migration by themselves.

The Cx protein family has 21 members in humans and 20 in mice, which are named according to the molecular weight of the protein. Their functions and distribution in cells and tissue are ubiquitous. The docking of hemichannels allows the passage of small ions, metabolites and messenger molecules. While not all channels are the same, they all share the property that the molecules must be less than 1 kiloDalton (kDa) in size.

Cx assemble in hexamers and as such, they are transported to the plasma membrane via the endoplasmic reticulum to create a hemichannel, which can dock with hemichannels on adjacent cells. Clusters of these hemichannels assemble to make a GJ. During their life cycle, other binding proteins are involved in the regulation of Cx assembly and trafficking, membrane insertion and GJ plaque formation. Binding proteins include cytoskeleton components such as microtubules and actin, which support transport to the cell membrane; adherens junction components such as cadherins, α-catenin, and β-catenin, which help in adhesion of hemichannels; tight junction proteins, zonula occludens-1 and -2, which aid in the organization and trafficking of GJ; and enzymes such as kinases and
phosphatases, which regulate the assembly, function, and degradation of hemichannels.\textsuperscript{30, 31}

EC express at least three Cx: Cx37, Cx40 and Cx43, while hematopoietic cells seem to only express Cx43.\textsuperscript{35, 36, 37} Cx43 is encoded by the GJ protein alpha 1 gene (\textit{Gja1}). Cx43 contains four transmembrane domains, two extracellular loops containing six conserved cysteine residues, a cytoplasmic loop and cytoplasmic N- and C- termini.\textsuperscript{30, 38} It is the most widely distributed Cx in tissues and has been shown to be the predominant Cx expressed in hematopoietic tissue. Partial loss-of-function mutations in Cx43 induce human disease. The most relevant example is a relatively rare disease called oculodentodigital dysplasia (ODDD), which is characterized by enchondral ossification defects.\textsuperscript{39} The specific deficiency of Cx43 in EC induces hypotension and bradicardia associated with decreased levels of angiotensin and nitric oxide.\textsuperscript{40}

**Cx43 in the BM microenvironment**

Very little is known about the regulatory mechanisms of communication in the hematopoietic microenvironment. However, it has been well documented that Cx43 expression is critically important to the BM microenvironment.\textsuperscript{9, 36, 41, 42, 43} Cx43 modulates osteoblast differentiation and function, and is required for normal skeletal development and bone remodeling throughout life.\textsuperscript{44} Several studies have shown that Cx43 is expressed on osteoblasts and stromal cells. In studies using knockout (KO) mice models where the \textit{Gja1} gene has been deleted, Cx43 GJ have been shown to contribute and participate in the physiology of hematopoiesis.\textsuperscript{36, 45}

Cx43 expression on stromal cells, a major component in the BM microenvironment, plays a critical physiological role in the support, regulation and
recovery of HSC/P after stress is induced.\textsuperscript{41} Stromal-stromal cell communication has been shown to be important for providing an optimal BM microenvironment for HSC/P using electrophysiological and dye coupling techniques.\textsuperscript{43, 46} Studies, performed by Rosendaal et al, have also shown that under basal conditions, GJ are rarely found, however the use of 5-FU chemotherapy induces over-expression of BM Cx43 \textit{in vivo}. This group observed that in response to forced active division of stem cells due to myeloablation, Cx43 expression is increased 100-fold in the BM microenvironment and coupled to HSC.\textsuperscript{42} Our group has previously demonstrated that the deficiency of Cx43 in both HSC/P and stromal cells causes a decrease in progenitor populations after 5-FU treatment. This study showed that hematopoietic recovery after 5-FU treatment is severely impaired in Cx43 deficient mice compared to WT mice. Pre-chemotherapy blood samples were taken and complete blood counts (CBC) were performed on both Cx43 deficient and WT mice, with results showing no difference in cell counts between groups under these basal conditions. However, by 14 days post-5-FU-therapy, Cx43 deficient mice had been unable to recover from their pancytopenia compared to WT mice. Competitive transplantation of Cx43-deficient HSC/P from 5-FU-treated primary mice, where Cx43 deficiency had been induced in adulthood, showed that Cx43 deficiency induces a failure of long-term HSC to develop a normal hematopoiesis. This experiment indicated that Cx43 is critical in active hematopoiesis.\textsuperscript{10} Together, these studies provide evidence that Cx43 expression within the BM is crucial in the development of an efficient response to hematopoietic stress.

Cx43 is the most abundantly expressed Cx on vascular EC, however, it is not known whether BMEC expressing Cx43 play a major role in supporting HSC through intercellular communication as it does in stromal cells. Recent findings from our
laboratory at Cincinnati Children’s Hospital Medical Center (CCHMC, Drs. Jose Cancelas and Kyung Hee Chang, personal communication) have shown that Tek-Cre;Cx43 deficient mice have ~3-fold more progenitors in their PB than WT mice, suggesting that endothelial Cx43 expression may play a role in retaining HSC/P in the BM vascular niche. We hypothesized that a loss of Cx43 expression in EC may cause decreased adhesion of HSC/P in the hematopoietic microenvironment and/or “loose” connections between sinusoidal EC, impairing the retention barrier function of sinusoidal EC, and allowing more progenitors to migrate to the PB. Based on this information, we proposed an in vitro study with isolated murine EC from the BM stroma to investigate the role of Cx43 in BMEC. This project intends to provide further insight on the physiological role of BMEC Cx43 GJ in HSC function in adult hematopoiesis using systematic analysis of endothelial-specific gene disruption of Cx43.
Figure 1. Model of the vascular niche. Endothelial Cx43 GJ form between adjacent BMEC and/or HSC/P and play a critical role in HSC/P adhesion and migration.

**HYPOTHESIS**

Cx43 expression on BM derived EC regulates HSC/P adhesion and/or migration to and from the vascular niche (Figure 1). Loss of BM endothelial Cx43 expression induces intrinsic endothelial cell impairment, which translates into changes in EC proliferation, survival, HSC/P adhesion to the BM endothelium and/or HSC/P transendothelial migration through the BM microvasculature.
RESEARCH PLAN

This project intends to analyze the role of BMEC Cx43 GJ in the regulation of BMEC proliferation and on the migration of HSC/P to and from the vascular endothelium in the BM. Two methods were used to generate Cx43 deficient EC: 1) Isolation of Tek-Cre;Cx43 WT or Tek-Cre;Cx43flox/flox BMEC; 2) Interference of endogenous Cx43 expression through transduction and exogenous expression of Cx43 short-hairpin ribonucleic acid (shRNA) in cultured murine endothelial progenitor cells (mEPOC). These two methods will allow us to distinguish between cell-specific changes induced by loss of Cx43 expression (by gene interference) and possible changes in the BMEC composition induced by embryonic and postnatal Cx43 gene deletion. Cx43 gene disruption was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) and protein immunoblot analysis. Cx43 KO and WT EC were then characterized and used for transendothelial migration and adhesion in which either WT or KO HSC/P were added.

SPECIFIC AIMS

1. To obtain and characterize BMEC derived from Tek-Cre;Cx43 WT or Cx43flox/flox mice and mEPOC (available commercially) transduced with shRNA to interfere with Cx43 expression.

2. To analyze the physiological role that Cx43 GJ plays in controlling BMEC proliferation.

3. To determine if endothelial Cx43 GJ control HSC/P adhesion and migration using adhesion and transendothelial migration assays.
MATERIALS AND METHODS

Deletion of the Gja1 (Cx43) Gene

Method A: Conditional Knockout Gene Deletion Model (In Vivo)

A conditional KO gene deletion model was used because mice with homozygous Cx43 null alleles die at birth due to cardiac complications. To induce Cx43 gene deletion the Cre/loxP KO method was used. Cre is a transgenic recombinase protein, originally from the bacteriophage P1, introduced as a transgene through electroporation in the germinal cell line used to generate Cre-transgenic mice. Cre recognizes site-specific recombination and catalyzes the recombination of DNA between two loxP sites and the target gene. A loxP site is an engineered sequence consisting of two 13 base pair (bp) inverted repeats (a palindrome) separated by an 8 bp asymmetric spacer region. The mouse strain containing the target gene (in this case Cx43) is flanked by the two loxP sites and is called the “floxed gene”, which has been introduced into the mouse genome of the transgenic mouse through homologous recombination. As a result of the Cre recombinase activity, the gene located between the two loxP sites is excised. To induce endothelial specific Cx43 gene deletion, the promoter Tek (Tie2), which activates transcription of EC specifically, was used to control the Cre-recombinase activity. Tek-Cre;Cx43 WT transgenic mice were crossed with Cx43flox/flox, non-Cre mice by our laboratory. The third and subsequent offspring generations that were Cx43flox/flox-transgenic (Tek-Cre KO) or single Tek-Cre transgenic mice without the floxed Cx43 gene (Tek-Cre WT) were used in our experiments as determined by PCR. To induce hematopoietic-specific Cx43 deletion, Vav1-Cre;Cx43 WT or
Cx43\textsuperscript{flox/flox} transgenic mice (Vav1-Cre WT or KO) were used in the same fashion. An example of each phenotype, including transgenic WT and transgenic flox/flox mice, resulting from this breeding strategy are shown in Figure 2 (genotyped and kindly provided by Susan Dunn). Protocols followed were approved by the animal facility at CCHMC using ethical guidelines by the Institutional Animal Care and Use Committee (Protocol 7D04033 PI: Dr Jose A. Cancelas) and all the work was performed within the Institutional Biosafety Committee for BSL1 and BSL2 laboratories (Protocol 7DAV04020 PI: Dr. Jose A. Cancelas).

Method B: shRNA Gene Knockdown Model (In Vitro)

Figure 2. Genotyping by PCR of Cx43 transgenes. All possible genotypes from breeding strategy for Tek-Cre and Vav1-Cre mice are included. Het Ntg represents heterozygous Cx43\textsuperscript{flox/WT}; non-transgenic mice. F/F Ntg represents Cx43\textsuperscript{flox/flox}, non-transgenic mice. WT Tg represents Cx43 WT transgenic mice. Het Tg represents heterozygous Cx43\textsuperscript{flox/WT}; transgenic mice. F/F Tg represents Cx43\textsuperscript{flox/flox}; transgenic mice. WT Ntg represents Cx43 WT; non-transgenic mice. Genotypes boxed in red were used for experiments. Cx43 flox amplicon length: 220 bp. WT amplicon length: 180 bp. Cre amplicon length: 150bp. (Figure adapted from experiment performed by Susan Dunn).
RNA interference using shRNA is an alternate method used to knock down gene expression in vitro. ShRNA, a short sequence (~20-25 nucleotides) homologous to the target gene to be knocked down, is delivered into the cells with the use of a retrovirally-encoded vector and is an effective means of triggering stable gene silencing using the RNA inference pathway in cell lines.\textsuperscript{48} The shRNA is recognized by an enzyme called dicer, an RNase III-like enzyme, which cleaves the shRNA precursors into short interfering single stranded RNA, and the protein-RNA effector nuclease complex called RISC (RNA-induced silencing complex) then binds the target DNA. RISC acts as a platform upon which different regulatory modulators may attach, causing either mRNA degradation (for sequences targeting the open reading frame of the target complementary DNA (cDNA)) or translational inhibition (for sequences homologous to the 3′-untranslated region) of the target RNA sequence.\textsuperscript{49}

A commercially available cell line, mEPOC (7030031, Biochain, Hayward, CA) was used for this method of gene disruption. mEPOC had been isolated from the BM of 7-8 week old C57/B6 mice and further cultured and tested for known EC markers by PCR by the manufacturer. They were delivered at the ninth passage as cryopreserved cells. Kyung Hee Chang, PhD, thawed and plated the cells for culture according to manufacturer’s instructions. Before the mEPOC cells were shRNA transduced, they were transduced with SV40 (simian virus large T antigen, see SV40 Transduction section of materials and methods), allowing them to grow for indefinite use.
mEPOC were then plated, grown and transduced with three types of lentiviral particles. Each of the lentiviral particles contained a plasmid with a different oligonucleotide sequence that targeted a homologous sequence of the Cx43 gene and a puromycin resistant gene (all from Sigma-Aldrich, St. Louis, MO) that generated siRNA intracellularly at a MOI (multiplicity of infection) of 20. Eight µg/mL of hexadimethrine bromide (H9268, Sigma-Aldrich) was used to ensure efficient uptake into the cells. The oligonucleotides contained in the plasmids contained by the three types of lentiviral particles were:

1) a non-target control sequence; (called shRNA/control, CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCT TGTGGTTTTTG);

2) a 3′ untranslated region on exon 2 of the Cx43 gene target sequence, (shRNA A, CCGGCCCACCTTTGTGTCTTCCATACTCGAGTATGGAA GACACAAAGGTGGGTTTTG); and

3) an open translated region on exon 2 of the Cx43 gene target sequence, (shRNA E, CCGGGCTCTTCTATGTCTTCTTCAACTCGAGTTGAAGAAG ACATAGAAGAGCCTTTTTG).

After 16-24 hours the media containing the lentiviral particles was removed, and fresh media was added. The following day, previously titered puromycin concentrations (1.0µg/mL, representing the minimal concentration of puromycin able to kill 100% of untransduced cells) were added to the cells in culture. Cells that did not uptake the viral particles died, and after 10-12 days only cells containing shRNA remained and were expanded. PCR and western blot were used to determine the amount of target (Cx43) gene expression in each mEPOC line.
Development of Tek-Cre Transgenic BMEC Lines

First, BMEC lines were established using murine BM cells extracted from Tek-Cre WT and Tek-Cre KO mice (as described in Method A). The mice were sacrificed and whole BM cells were obtained from femurs, tibias and iliac crests. The extracted cells were crushed, filtered and red blood cells were lysed using 1x RBC lysis buffer (BDPharmlyse 555899, BD Bioscience, San Jose, CA) Cell dissociation buffer (13151-014, Invitrogen, Carlsbad, CA) was then added to the resuspended cells and incubated for 15 minutes in 37°C water bath to further break apart the EC from the microvasculature. The BM cells were then explanted to tissue culture and grown on a matrix containing EC enhancement media. The cell culture flask was coated with fibronectin (F4759, Sigma-Aldrich), previously incubated at 37°C for 2 hours, in order for the cells to adhere. The media used was specific to aid EC growth and prevent bacterial contamination. It consisted of Dulbecco’s modified Eagle’s medium (DMEM/F12; Invitrogen), 10% fetal calf serum (FCS; Invitrogen), 15% EC growth supplement (ECGS; Invitrogen), 15% ITS (insulin, transferrin, selenium; Invitrogen), 2% L-glutamine (Invitrogen), and 2% penicillin and streptomycin (Invitrogen). Cells were passed upon subconfluency (~80%) for a minimum of 2 passages. After several passages, the cells were characterized using flow cytometry, and immortalized using SV40 transduction. Once the cells were immortalized, they were sorted for known EC phenotypic markers using flow cytometry (FACS Aria II, BD Biosciences) in order to obtain a heterocellular endothelial cell line. Once the EC were established, Cx43 expression in the WT and KO cell lines was determined using PCR and western blot analysis.
**SV40 Transduction**

In culture, primary cells can be cultured a limited number of passages before they undergo growth arrest and irreversible senescence (lose the ability to divide). SV40 transduction is one method used to keep cells in a proliferating state. SV40 causes cell transformation by disabling the retinoblastoma protein (Rb) and p53-tumor-suppressing pathways, allowing cells to proliferate indefinitely. Rb and p53 are transcription factors that, when activated, prevent cell mutation or excessive cell growth by inhibiting cell cycle progression and initiating apoptosis of deoxyribonucleic acid (DNA) damaged cells. However, it is known that certain stromal cells in culture have the ability to spontaneously inactivate p53-activation after a long series of cell passages, causing these stromal cells to continue to divide. We hypothesized that our EC would also undergo this spontaneous mutation, but for the time constraints of this project, SV40 transformation was necessary to allow immortalization of these cells. There are some possible difficulties in using SV40 transformation that may include modifying other cellular functions downstream of the p53 pathway, but other studies show there is no difference in cell function between SV40 transformed and non-transformed cells (observations by Dr Robert Oostendorp, Erasmus University of Rotterdam, as reported by Dr. Jose Cancelas).

In order to immortalize the EC in culture, a retroviral plasmid designated Linker was utilized (kindly donated from Dr Susan Wells’s laboratory, CCHMC). The plasmid contains a cytomegalovirus promoter, SV40 cDNA, and a neomycin resistant gene that, when transduced into cell culture, would infect the cells and begin replication. Retroviral plasmid medium was added at a 1:1 ratio in addition to 8µg/mL of hexadimethrine bromide to EC in culture plates (concentration of the viral
particles was unknown). The plates were incubated at 37°C for 4 hours, washed, and new EC media was added. Plates were allowed to grow and upon subsequent confluence, cells were passaged. No neomycin was needed to favor the outgrowth of SV40 transformed BMEC.

**Protein Expression Analysis**

Cx43 deficiency was tested by western blot analysis using whole cell lysate or membrane bound expression. For total cell lysate, approximately 300,000 EC from each source were obtained from culture, washed with phosphate buffered saline (PBS) and pelleted in a microtube. EC were then lysed with an addition of ice-cold lysing buffer (RIPA 9806, Cell Signaling Technologies, Boston, MA), and protease inhibitors (11697498001, Roche, Indianapolis, IN) according to the manufacturer’s instructions. The protein concentration was determined by performing a bicinchoninic acid (BCA) protein assay according to manufacturer’s instructions (23225, Pierce, Rockford, IL). Approximately 10 μg/μL of protein was then resuspended with 1:1 dilution of Laemmil’s buffer (161-0737, BioRad, Hercules, CA) heated at 95°C for 5 minutes, and then cooled on ice for 15 minutes. The protein content of the samples was separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis, 10% polyacrylamide) for 1 hour at room temperature and transferred onto a nitrocellulose membrane by electroblotting for 1 hour at 4°C. The membrane was washed 3 times at 10 minute increments with a working solution (TBS-t) containing, 20mmol/L Tris buffered saline (TBS T5912, Sigma-Alrich) and 0.1% Tween-20 (wt/vol P5927, Sigma-Alrich), and incubated for one hour in 5% nonfat milk powder in TBS to block nonspecific sites. It was then rinsed an additional 3 times with TBS-t and incubated overnight with polyclonal or monoclonal anti-Cx43
(710700 or 138300 respectively, Invitrogen). The next morning, the membrane was washed 3 times with TBS\(t\) and incubated with secondary antibody (anti-rabbit 7074, or anti-mouse 7067, immunoglobulin horseradish peroxidase conjugate, Cell Signaling Technology) and the bands were visualized with a chemiluminescence detection system according to manufacturer’s instructions (LumiGLO 7003, Cell Signaling technology, or Femto Maximum Sensitivity Substrate 34095, Thermo Fisher Scientific, Waltham, MA). Monoclonal β-actin antibody (A5441, Sigma-Aldrich) was used as a loading control to semi-quantify the concentration of protein used in each lane. (β-actin is a house keeping protein in which all cells express the same number of gene copies.) In addition, protein from cell lines known to express and not express Cx43 were run in parallel as a control for the location of the bands, in addition to running a loading biotinylated ladder.

For Cx43 membrane bound expression, \(1 \times 10^6\) cultured BMEC cells were pelleted in a microtube, briefly rinsed with cold calcium/magnesium-free PBS and then lysed by an addition of ice-cold alkaline buffer, containing 2mmol/L NaHCO\(_3\), 20mmol/L NaOH, 5 mmol/L ethylenediamine tetraacetic acid (EDTA) and protease inhibitor, according to the manufacturer’s instructions\(^\text{10}\). The lysate was then sheared by serial passage through a 25-gauge needle and further purified by 3 serial cycles of liquid nitrogen freezing and thawing in a 37°C water bath. The lysate was then incubated on ice for 30 minutes. An aliquot was saved to determine protein concentration by BCA protein assay (Pierce). The remaining sample was then ultracentrifuged (Optima ultracentrifuge, SW55Ti, rotor 342194, Beckman Coulter, Brea, CA) at 148,600 x \(g\) for 1.5 hours at 4°C. The samples were then resuspended with 1:1 dilution of Laemmil’s buffer and heated at 95°C for 5 minutes. Protein
expression was then determined using the method above, starting with protein separation by SDS-PAGE.

**Gene Expression Analysis**

RT-PCR was used to confirm the presence or absence of Cx43 gene expression. Total RNA was extracted from shRNA transduced mEPOC or Tek-Cre;WT or KO EC from culture using a commercial reagent solution (TRI Reagent AM9736, Ambion, Austin, TX) using the TriPure Isolation protocol (Roche Applied Science). RNA concentration was measured spectrophotometrically at 260 nm (Nanodrop 2000, Thermo Fisher Scientific, Waltham, MA) and a minimum concentration of 100ng/µL RNA was used. The RNA was used as a template for single-strand cDNA synthesis using the Taqman system, following manufacturer’s instructions (Taqman Reverse Transcription Reagents N808-0234, Applied Biosystems/Invitrogen, Carlsbad, CA). Two µL of RNA (approximately 100ng/µL), were added to Taqman master mix, and made up to a final volume of 30µL per sample with RNase free water (UPW0125, MBI/growcells.com, Irvine, CA). Thermal cycling parameters for reverse transcriptase amplification included a 10 minute at 25°C incubation period, 30 minutes at 48°C for RT amplification, and 5 minutes at 95°C for strand separation and RT inactivation (BioRad C1000, Hercules, CA). A solution containing SYBR green (SYBR GREEN PCR Master Mix 4309155, Applied Biosystems/Invitrogen; used according to manufacturer’s instructions), together with 4.5µmol/L of each of the forward and reverse primers, was added to 2 µL synthesized cDNA to for a final volume of 25µL. The oligonucleotide primers used were as follows: mouse Cx43-forward, TTGACTCAGCCTCCAAGG; mouse Cx43-reverse, AATGAACAGCACCAGACGC; glyceraldehyde-3-phosphate
dehydrogenase (GADPH, a housekeeping protein)-forward, GTATGACTCCACTCACGGCAA; reverse, CTAAGCAGTTGGTGGTGCAG, (all from Invitrogen). Amplification was performed using a thermal cycler (BioRad C1000) under the following conditions: denaturation at 95°C for 5 minutes, annealing and extension to primers at 95°C for 30 seconds, and at 60°C for 1 minute for 40 cycles, followed by a final extension at 60°C for 10 minutes. The controls used to test for the integrity of the RNA preparation were the expression of GADPH, 2 µL RNAse free water (MBI/growcells.com), and commercially prepared mouse heart DNA (Invitrogen) known to express Cx43. To control for the specificity of the primers, samples were run without reverse transcriptase as well. Electrophoresis was then performed on PCR products using a 2-3% (wt/vol) agarose gel. Bands were visualized at 172 bp amplicon length for Cx43 and 320 bp amplicon length for GAPDH using ethidium bromide staining and photographed under ultraviolet light.

A semi-quantitative analysis of the protein or gene level of Cx43 expression was performed using densitometric analysis. The density from each band was calculated (ImageJ software, US National Institutes of Health, Bethesda, MD). The Cx43 band density was divided by the housekeeping protein (β-actin or GAPDH) loading control band density for each cell line and normalized to the concentration determined from the shRNA/control or Tek-Cre WT Cx43 expression, respectively.

**Flow Cytometry Analysis for EC Phenotypic Characterization**

Explanted EC were characterized using the following phenotypical fluorochrome-conjugated rat anti- mouse antibody markers: phycoerythrin (PE) - conjugated anti-VEGFR-2 (555308, clone Avas12α1,BD Bioscience); APC (allophycocyanin)- conjugated anti-CD34 (560230, clone RAM34, BD Bioscience);
FITC (fluorescein isothiocyanate)-conjugated anti-CD106 (553332, clone 429MVCAM.A, BD Bioscience); 7-aminoactinomycin D (a marker of live cells; 7-AAD A1310, Invitrogen, Molecular Probes) and PE-cyanan 7-conjugated anti-CD45 (also known as leukocyte common antigen, 552848, clone 30-F11, BD Bioscience). EC were resuspended in 100µL of 2% mouse serum/PBS and stained with the fluorochrome-conjugated IgG antibodies or their isotype control (for each cell line respectively.) Separately, primary monoclonal unconjugated rat anti-mouse Tie2 (Tek) antibody (MAB1148, Millipore) was also used to stain the EC. The cells were incubated for 15 minutes, washed and a secondary fluorescein conjugated goat anti-rat IgG antibody was added. After 15 minutes, all cells were washed with PBS and analyzed by flow cytometry (FACS Canto, 6-color, 2-laser (Ar 488 nm and HeNe 633 nm) BD Biosciences). Flow cytometry analysis was determined (FloJo software v 7.5.5, Treestar Inc. 2009, Ashland, OR). Median fluorescence intensity (MFI) ratio was calculated as the median fluorescence channel for each surface antigen analyzed, divided into the MFI of the isotype-labeled control for each cell line. A change greater than 2-fold in the MFI ratio between cell lines was considered significant.

Cell Proliferation and Survival During Culture Analysis

EC proliferation was performed by counting cells from culture at different time points using a trypan blue (T8154, 0.4% wt/vol, Sigma-Aldrich) dye exclusion test. Trypan blue is a vital stain used to selectively color non-viable tissues or cells blue. According to the package insert, live cells or tissues with intact cell membranes do not allow the entry of trypan blue into their cytoplasm. In order to determine the proliferation ability of EC deficient in Cx43 compared to control EC, a 10 day study
was performed using trypan blue staining on shRNA transduced mEPOC cell lines. On Day 0, 25,000 EC were added to 6-well tissue culture plates and expanded. Every 48 hours the cells were trypsinized and resuspended in 1mL of PBS. Ten µL of cell suspension was stained with 10µL of trypan blue and both live and dead cells were counted per manufacturer’s instructions. The experiment was performed in triplicate for each time point.

**Hematopoietic Colony Forming Unit-Content (CFU-C) Assay**

The CFU-C assay is an *in vitro* system used to quantify HSC/P in culture. Semi-solid methylcellulose culture medium containing 1% IMDM (Iscove’s modified Dulbecco’s medium), 30% FCS, 50 ng/mL recombinant mouse-SCF (stem cell factor), 3U/mL recombinant human-erythropoietin, 10 ng/mL recombinant human-interleukin (IL)-3 and IL-6 (Methocult3434, StemCell Technologies, Rocky Hill, NJ), and 1% penicillin/0.1 mg/mL streptomycin was utilized for transendothelial migration and adhesion assays. Total low density BM (LDBM), consisting of HSC/P, which migrated to the bottom of the transwells or adhered to the EC in culture, were extracted, resuspended in 200µL of EC media, added to the 3.8 mL of methylcellulose, vortexed, and plated on 35mm CFU-C dishes. After 8-9 days, the CFU-C were observed and counted on the input and output cell fractions, as previously published.57

**Adhesion Assay**

Endothelial-HSC/P cell adhesion plays a major role in cellular communication and regulation, and is of fundamental importance in the development and maintenance of tissues. CAMs and other direct cell-cell contact proteins such as GJ
are important mediators of leukocyte recruitment and adherence to the endothelium. In order to study the role of Cx43 GJ on EC, an in vitro cell adhesion assay was used. In 24-well tissue culture plates, 2 x10^5 mEPOC were plated in triplicate. Upon 100% confluency, the cells were irradiated at 12 Gray (unit of absorbed radiation energy). 40,000 Vav1-Cre WT LDBM cells were added to the plates and incubated at 37°C for 3 hours. After the respective time point was reached, the supernatant was removed and the non-adherent cells were gently washed away with PBS. Adherent cells were then harvested by vigorously rinsing the plates with 0.05% Trypsin-EDTA (wt/vol 25300, Invitrogen) for 5 minutes at 37°C. The extracted LDBM cells were then plated using the CFU-C assay as described above. The percentage of adhered HSC/P was calculated as followed:

\[
\text{Output (number of colonies/40,000 cells)} \times 100 = \% \text{ adhesion} \\
\text{(Input number of colonies/20,000 cells plated)} \times 2
\]

**Transendothelial Migration Assay**

Transendothelial migration of Vav1-Cre;Cx43 WT or Vav1-Cre;Cx43 \(^{\text{flox/flox}}\) murine derived HSC/P through Tek-Cre WT or KO EC or shRNA transduced mEPOC were analyzed in transwells (24-well plate 3422, Corning Inc., Corning, New York) with a gradient of CXCL12 (100 ng/mL) added to the bottom of the wells using a previously established protocol.\(^{58}\) Briefly, on established 100% confluent monolayers of BMEC cells, adherent to 6.5mm polycarbonate transmembrane inserts of 8-μm pore diameter (3422, Corning Inc.), a total of 50,000 Vav1-Cre WT or Cx43-deficient LDBM cells were deposited. After 16 hours, the migrated LDBM cells were extracted from the bottom of the transwells and plated using the CFU-C assay.
as described above. The assay was performed in triplicate for each EC type on from two separate experiments, and results were combined. The percentage of transmigrating HSC/P was calculated as followed:

\[
\text{Output (\# colonies/50,000 cells) } \times \frac{100}{\text{Input \#colonies/20,000cells plated}} \times 2.5 = \% \text{ transmigration}
\]

**Statistical Analysis**

Results were expressed as the mean ± standard deviation except where otherwise noted. The Student unpaired t-test was used to compare two independent samples. The statistical test and statistical significance threshold was established at \( p < 0.05 \) unless otherwise noted (Excel 2003, v.11.0.8307.0, Microsoft Corporation, Seattle, WA).
RESULTS

In order to characterize the role of Cx43 on BMEC in relation to hematopoiesis, we investigated the ability of BMEC to proliferate and their ability to adhere and allow transendothelial migration of HSC/P. For that reason, isolated and expanded BMEC (developed as indicated in Material and Methods) were phenotypically characterized for antigens expressed by EC and endothelial precursors, analyzed for Cx43 expression at the RNA and protein levels, and analyzed for their proliferation and survival along with their ability to allow adhesion and transendothelial migration of HSC/P.

Immunophenotype of BMEC

Previous studies have shown that EC and precursors are positive for cell surface antigens: Tie2, an EC marker; KDR (VEGFR2), an EC growth factor receptor; CD106 (VCAM-1), an adhesion molecule receptor; CD34, a hematopoietic and EC progenitor marker; and negative for CD45, a pan-leukocyte marker not expressed by EC or their precursors, whereas stromal cells, not derived from hematopoietic origins, are CD106+/CD34−/CD45− when immunofluorescently analyzed by flow cytometry. Based on these markers, the Tek-Cre WT and KO BMEC were sorted in order to further isolate the EC population from other cells in culture (mEPOC did not require cell sorting as they were previously established as EC by the manufacturer as described in Method B in Materials and Methods). Two serial steps of cell sorting were performed to select for CD45− cells, which was successful in removing macrophages and other leukocytes from culture, followed by a second cell sorting for the expression of CD106+/CD34+ cells (data not shown).
After using either method A or B (described in Material and Methods), putative EC were analyzed immunophenotypically for all four positive markers described above. (Figures 3 and 4 and Table 1). All cell lines expressed antigens Tie2, KDR, CD34 and CD106 and were composed of immunophenotypically homogenous cell populations using multicolor flow cytometry analysis. The MFI ratio was used to compare homogenous populations of cells with respect to the phenotypic antibody markers that are used for staining. It was observed that the MFI ratio for CD34 and CD106 expression in Tek-Cre KO cells, obtained through Method A in Materials and Methods, was over 2-fold higher than their WT counterpart cell line, suggesting that the content of endothelial progenitors may be increased in the Tek-Cre KO cell line. These results are compatible with the findings obtained by Daniel Gonzalez-Nieto, PhD (manuscript in preparation) in which the BM of collagen1A1 (Col)-Cre Cx43 deficient mice (Cx43 deficient in BM stromal (mesenchymal and osteoblast cells)) contains a significantly increased number of mesenchymal progenitors. Detailed analysis on a larger number of murine-derived EC lines is needed to confirm whether these results are similar to those found by Dr Gonzalez.
Figure 3. Expression levels of surface proteins on Tek-Cre EC (WT and KO) and shRNA transduced mEPOC (A, E, and shRNA/c) cell lines. Black line = isotype control. Blue line = antibody marker. There were no major differences in the expression profile of mEPOC cell lines, which were positive for the four antigens tested. Tek-Cre KO cells over-expressed (>2-fold) CD34 and CD106 antigens.
Figure 4. Expression of double surface antigens on Tek-Cre (WT and KO) EC and shRNA transduced mEPOC (A, E, and shRNA/control) cell lines. All cell lines seem to be composed of a single cell population with regard to the expression of the three relevant antigens used to define EC progenitor populations.
Table 1. Expression of surface antigens, CD34, CD106, Tie2 and KDR, for Tek-Cre (WT and KO) EC cells and shRNA transduced mEPOC (A, E, and shRNA/control) cell lines (MFI ratio).

<table>
<thead>
<tr>
<th></th>
<th>Tie2-FITC</th>
<th>KDR-PE</th>
<th>CD34-APC</th>
<th>CD106-FITC</th>
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<tr>
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<td>1.9</td>
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<td>2.1</td>
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<tr>
<td>Tek-Cre KO Ratio</td>
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<td>2.3</td>
<td>7.0</td>
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<tr>
<td>mEPOC A Ratio</td>
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<td>2.6</td>
<td>4.7</td>
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<tr>
<td>mEPOC E Ratio</td>
<td>2.9</td>
<td>2.3</td>
<td>1.3</td>
<td>7.3</td>
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<tr>
<td>mEPOC shRNA/c Ratio</td>
<td>2.6</td>
<td>2.6</td>
<td>2.3</td>
<td>4.3</td>
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Cx43 Expression of mEPOC and Tek-Cre BMEC cells

The expression of Cx43 was analyzed in Cx43 shRNA transduced mEPOC cells lines using western blot and RT-PCR. As indicated in Material and Methods, two different oligonucleotides (A and E) and a non-target control (ShRNA/c) cloned in lentiviral vectors containing a puromycin-resistance gene were used in a transduction protocol of EC. These two oligonucleotides were chosen based on reported silencing efficiencies of approximately 95% and 70%, respectively, in murine mesenchymal stem cells (Dr. Daniel Gonzalez-Nieto, personal communication). After the initial shRNA transduction and addition of 25µg/mL puromycin, we observed that the Cx43 expression of shRNA A was ~90% decreased compared with the shRNA/control cells, while the cells transduced with the shRNA E showed a higher level of Cx43 expression than the shRNA control-transduced cells and WT mEPOC that were not transduced (Figure 5). In order to confirm that the
Cx43 levels found in shRNA E were also present on the EC membrane, western blot analysis was performed using the Cx43 membrane bound protocol described in the Material and Methods. Using this method, shRNA E showed a 2-fold increase in Cx43 expression compared to the shRNA/control, which is considered to express normal levels of Cx43 protein (Figure 6). We were unable to determine if the over-expression of Cx43 was caused by the transduction of shRNA E clone into the mEPOC cells. It is possible that differences between stromal cells and EC post-transcriptional factors, present during RISC binding of the target Cx43 sequence, will cause differences in Cx43 expression. In addition, this discrepancy could be due to some type of protein interference or transduction issue in the shRNA control cells, causing them to express less Cx43, since their expression level of Cx43 was reduced compared with that of untransduced WT cells (Figure 5).
Figure 5. Western blot analysis of Cx43 expression from total lysate in mEPOC cell lines. WT represents non-shRNA transduced mEPOC Cx43 expression. shRNA/c, A, and E mEPOC are shRNA transduced with different clones (Materials and Methods). Cx43 expression ratio was calculated using densitometry of Cx43 bands divided into the housekeeping gene (β-actin) band density for each cell line, and normalized to the shRNA/control (shRNA/c) cell line.

Figure 6. Western blot analysis of Cx43 expression from membrane bound protein in shRNA transduced mEPOC cell lines. The Cx43 expression ratio was determined by dividing the calculated density of Cx43 bands into the density of the β-actin bands for each cell line. Bands were normalized to the concentration of shRNA/control (shRNA/c). Stromal cells known to express and be deficient in Cx43 were run in parallel as controls (results not shown).

The shRNA A interferes with RNA expression by binding the 3’ untranslated region of the RNA but not the coding sequence of the Cx43 cDNA. While the protein levels of Cx43 were drastically reduced in EC transduced with shRNA A, we observed no difference in the RNA expression levels between the non-target control
and the shRNA A-expressing cell lines (Figure 7). Additionally, we did not find any
difference in the RNA expression level of the shRNA E-expressing mEPOC cells
(Figure 7), suggesting that the difference in protein content is due to post-
transcriptional changes induced by the expression of the shRNAs.

![Image of RT-PCR analysis](image_url)

**Figure 7.** RT-PCR analysis using extracted RNA from shRNA transduced mEPOC cell

*Top panel:* Lanes 1-3 show the expression of Cx43 in the mEPOC cell lines. Lanes 4-6
show the absence of bands when run without the addition of reverse transcriptase (negative
control; amplicon length: 172 bp.) *Lower panel:* RNA content shows the expression of GAPDH
(housekeeping gene control; amplicon length 320 bp.) A commercially prepared mouse heart
control known to express Cx43 was used as a positive control (results not shown).

After immunophenotyping and cell sorting, expression of Cx43 was also
determined in the Tek-Cre;Cx43 WT and KO EC from mice using semi-quantitative
analysis of western blot and RT-PCR. Total lysate from Tek-Cre;Cx43 KO EC in
culture showed a ~70% decrease in Cx43 protein expression compared to WT cells
(Figure 8). RT-PCR also confirmed the loss of Cx43 expression in 622KO EC from
culture, with a ~60% reduction in Cx43 expression (Figure 9).
Figure 8. Western blot analysis of Cx43 expression from total lysate in Tek-Cre WT and KO EC after cell sorting. Cx43 expression was normalized to WT Cx43 expression after the density concentration ratio was performed, in which Cx43 band density was divided by the β-actin band density for each cell type. (Analysis performed by Kyung Hee Chang, PhD).

Figure 9. RT-PCR analysis of Tek-Cre WT and KO EC. Left upper panel: Shows the expression of Cx43 in the presence of reverse transcriptase. Right upper panel: Shows the absence of bands when run without the addition of reverse transcriptase (negative control). Lower panel: Shows the expression of GAPDH (housekeeping gene control; amplicon length: 320 bp.) A commercially prepared mouse heart control known to express Cx43 was used as a positive control (results not shown). The Cx43 expression ratio was calculated densitometrically. The density of the Cx43 bands were divided into that of the house keeping gene band for each cell line and normalized to the WT cell line.
Due to the differences in immunophenotype and the incomplete deletion of Cx43 in BM cells derived in this experiment from Tek-Cre;Cx43\textsuperscript{flox/flox} mice, we did not perform any functional assay with the Tek-Cre;WT and Tek-Cre;KO cell lines.

**Cell Proliferation and Survival of shRNA Transduced mEPOC**

Cx43 has been shown to be responsible for the cell contact inhibition usually observed in non-transformed cell outgrowth *in vitro*. Cx43 deficiency, in fact, has been shown to induce a partial transformation phenotype where cell contact inhibition is lost.\textsuperscript{61} To understand whether EC Cx43 expression played any role in BMEC proliferation and possibly in their ability to create new vessels, we analyzed the proliferation and survival of EC in three mEPOC cell lines with very different levels of expression of Cx43. While mEPOC control cells expressed normal or low levels of Cx43, mEPOC A cells expressed very low levels of Cx43 and mEPOC E cells expressed high levels of Cx43. The proliferation rate of these three cell lines was determined by analysis of their cell expansion *in vitro*, when cultured in 6-well tissue culture plates for 9 days (Figure 10). On Day zero (initiation of the culture), the EC were not in contact with each other and extensions could be observed on the EC in culture. On Day 3 the EC were approximately 30% confluent and the number of EC in culture had expanded approximately 4-fold for all three cell lines. On Day 6, the cells had additionally expanded around 2-fold and were observed to be 60-75% confluent in culture. On Day 9, the shRNA control cells had additionally proliferated ~3.5 fold and were 100% confluent; however, mEPOC E cells expanded only 2.5 fold since Day 6. Interestingly, the mEPOC cell line, which overexpressed Cx43, showed a decreased proliferation when compared to the mEPOC cells transduced with shRNA A or shRNA control.
To rule out whether Cx43 expression controlled survival of BMEC, and therefore, whether the net effect on cell counts was due to increased cell death rather than decreased cell proliferation, we analyzed the cell death by trypan blue exclusion dye test. We observed that the level of expression of Cx43 did not seem to change their survival during *in vitro* culture (Figure11) These data are compatible with the role of Cx43 as a tumor suppressor able to block the proliferation of different cell types in culture.

**Figure 10. Proliferation study on shRNA transduced mEPOC cell lines.** EC were grown in culture for 9 days (in which confluency was 100%). Every 48 hours the number of live cells were counted (Materials and Methods). Solid circles represent shRNA/control cells. Empty circles with dashed line represents mEPOC A cells. Solid squares represent mEPOC E cells. On Day 9, shRNA/control EC proliferated significantly more than shRNA E EC, which were over-expressing Cx43. n = 3 per group. * p < 0.05.
Figure 11. Cell death study on shRNA transduced mEPOC cell lines. Number of dead EC counted using trypan blue every 48 hours (Materials and Methods). Empty circles represent shRNA/c cells. Solid circles with dashed line represents mEPOC A cells. Solid squares represent mEPOC E cells. Differences are not statistically significant. n = 3 per group.
Adhesion of Vav1-Cre WT Hematopoietic Progenitors to mEPOC cells

To determine the role of BMEC Cx43 expression on the adhesion of HSC/P when in contact with BMEC, we established an in vitro model of cell adhesion (Figure 12). After 3 hours of incubation, while the adhesion of HSC/P to Cx43-deficient mEPOC A or to Cx43-overexpressing mEPOC E cells was not significantly different from adhesion to mEPOC/control BMEC, the Cx43-deficient BMEC showed a modest but significant (15%) decrease in adhesion compared to Cx43 overexpressing BMEC (mEPOC E cells, p<0.05, Figure 13). These results suggest that the Cx43 overexpression may increase the ability of HSC/P to adhere to the BMEC.
Figure 12. Experimental design to study the effects of Cx43 deficiency using an adhesion assay. LDBM (containing HSC/P) was obtained from a Vav1-Cre; Cx43 WT mouse and added to the mEPOC monolayer and incubated for 3 hours. After 3 hours adherent HSC/P were extracted and CFU-C assay was performed. Hematopoietic progenitor colonies were counted as described in Materials and Methods.
Transendothelial Migration of HSC/P Through shRNA Transduced mEPOC Cell Lines

To elucidate the mechanism of Cx43 GJ on BMEC in the migration of HSC/P through the vasculature we used BMEC deficient in Cx43 obtained through Methods A or B (described in the Material and Methods section). We tested those lines in an in vitro model of transendothelial migration of HSC/P through BMEC monolayers in a gradient of the chemoattractant CXCL12 (Figure 14). CXCL12 is the most relevant chemoattractant in the BM, controlling the traffic of HSC/P to and from the BM microenvironment. We also analyzed whether HSC/P Cx43 expression controlled the transendothelial migration of HSC/P through WT or Cx43-deficient BMEC. Cx43
deficiency of HSC/P obtained from Vav1-Cre;Cx43\(^{\text{flox/flox}}\) mice was demonstrated by quantitative, real-time PCR (Q-PCR) on flow-cytometrically (FACS)-sorted lineage\(^{-}\)/c-Kit\(^{+}\) cells (LK cells) and multipotential hematopoietic progenitors (MPP, lineage\(^{-}\)/c-Kit\(^{+}\) /Sca-1\(^{+}\)/CD34\(^{+}\)/CD135\(^{+}\)), and compared with the same population isolated from Tek-Cre\(^{\text{transgenic; Cx43 WT BM (assayed by Dr. Daniel Gonzalez-Nieto; Figure 15). Transendothelial migration of WT HSC/P through Cx43-deficient EC (shRNA-A mEPOC) was increased by 22% compared with the shRNA/control and the Cx43-expressing shRNA-E mEPOC cell line. These results are compatible with the decreased retention of HSC/P found in the BM of Tek-Cre;Cx43 deficient mice and reported by Dr. Chang from our laboratory (unpublished data). Interestingly, the transendothelial migration of Cx43-deficient HSC/P was decreased an average of 25% compared with WT HSC/P, independently of the Cx43 expression level of the mEPOC cell lines, indicating that Cx43 expression in HSC/P is required for their full transendothelial migration (Figure 16). These results also suggest that HSC/P Cx43 function in transendothelial migration does not depend on homotypic Cx43-dependent channels.
Figure 14. Experimental design to study the effects of Cx43 deficiency using a transendothelial migration assay. Vav1-Cre WT or KO LDBM (containing HSC/P) was obtained and added to confluent WT or KO EC monolayer in a transwell. CXCL12 (100ng/mL) was used to attract the HSC/P, and after 16 hours, migrated cells were extracted and added to a culture dish for CFU-C. Hematopoietic progenitor colonies were counted after Day 8 as described in Materials and Methods.
Figure 15. Q-PCR analysis for Cx43 gene expression in Vav1-Cre WT and KO LK and MPP cell populations. Q-PCR measures the DNA product after every cycle. After the DNA is amplified, a cycle threshold in which fluorescence is first detected is obtained, and the relative gene expression is calculated. This value is divided by the housekeeping gene expression to determine the corrected fold increase of the DNA product. The solid bar represents Vav1-Cre WT cells and the empty bar represents Vav1-Cre KO cells. Results are from one experiment performed by Daniel Gonzalez-Nieto, PhD.
Figure 16. Transendothelial migration assay of Vav1-Cre WT and KO HSC/P through shRNA transduced mEPOC monolayers. There was a significant increase in the transmigration of WT HSC/P through Cx43 deficient EC (mEPOC A); however this increase was reverted by a deficiency of Cx43 in the HSC/P. Solid bar represents Vav1-Cre WT HSC/P. Empty bar represents Vav1-Cre KO HSC/P. Results are from two separate experiments combined, n=6/group, **p < 0.01.
DISCUSSION

An increasing number of studies have demonstrated the widening scope of the therapeutic potential of migration of HSC/P in stem cell collection, transplantation and tissue repair. Migration of HSC/P from the BM vasculature is a fundamental cellular function related to their ability to respond to stress and tissue regeneration, thereby allowing extramedullary expansion and repair. Extensive in vivo and in vitro research has shown that one of the most prominent factors initiating HSC migration is the chemokine CXCL12. It provides a gradient in which HSC/P recruitment to BM occurs within specialized marrow vessels that constitutively express vascular CD34, CD106, KDR and Tie2 receptors. Cx43 is present and involved in the migration of other cell types and is possibly involved in the migration process of HSC/P as well.

Normal adult hematopoiesis is controlled by the BM microenvironment within the HSC/P niche(s), composed of specialized cells including EC, mesenchymal progenitors, osteoblasts, adipocytes and extracellular matrix made of secreted proteins like collagen and fibronectin. Stem cells display a very active migration during fetal organogenesis. However, only hematopoietic and endothelial stem cells and progenitors have been clearly defined as tissue-specific stem cells with migratory activity during adulthood, keeping a homeostatic situation between vascular reservoirs in the BM, vessels and circulation. The movement of HSC/P does not only occur when damage is present, but is observed during homeostasis where a small number of HSC are constantly released into the circulation. This continuous migration depends on adhesive interactions of HSC/P to sinusoidal BMEC. The mechanisms controlling these two different populations and allowing
them to leave or return to the BM, are still very unclear.\textsuperscript{63,66} However, it has been
known for several decades that certain stimulators, such a granulocyte-colony
stimulating factor (G-CSF), are able to mobilize the HSC/P from the BM into the PB
by modifying the CXCL12 gradient between BM and PB.\textsuperscript{26,66}

Recently, it has been shown that adhesion molecules expressed by BMEC
like PECAM-1 influence steady-state HSC/P numbers in the PB. PECAM-1 deficient
mice have higher levels of HSC/P in the PB compared to their littermate controls.
PECAM-1 is required on both progenitors and BM vascular cells in order for efficient
transition between BM and PB, due to a decreased ability to migrate from blood into
the BM vascular niche in response to CXCL12.\textsuperscript{67} Unlike PECAM-1, CD34
expression in EC has been shown to mark a very different cell population. CD34 is a
cell-surface sialomucin widely used for HSC purification and as a marker of most
vascular EC, including those of capillaries in the majority of tissues and endothelial
progenitors. Recently, CD34 has been shown to function in leukocyte and HSC
trafficking through its action as a regulated blocker of cell adhesion and an enhancer
of migration.\textsuperscript{68}

Preliminary data from our laboratory (Drs. Cancelas and Chang, personal
communication) have shown that Tek-Cre;Cx43 deficient mice show an increased
number of HSC/P in the PB. We hypothesize that the transendothelial migration of
HSC/P is modified in the absence of Cx43 in vascular BMEC. Two alternatives are
possible to explain these results. First, that migration of HSC/P from BM to PB is
increased, or second, that migration of HSC/P from PB to BM is decreased. Since
Dr. Chang’s data suggested that these mice contain a modestly decreased number
of HSC/P in BM (personal communication), the most likely hypothesis is that Cx43
deficient BMEC fail to retain HSC/P in the BM and allow them to leak into circulation.
This increased migration may be due to decreased adhesion between BMEC and HSC/P, or through modifications in the chemoattractant gradients between BM and PB.

Cx43 has been shown to control migration of neural progenitors in the developing central nervous system through heterocellular adhesion between fibres from neuronal progenitors and radial glia, during fetal radial glial-guided migration. More recently, the same migration defect has also been demonstrated in neural progenitors. It apparently did not depend on decreased heterocellular adhesion but on a different mechanism involving the C-terminal domain of Cx43. Nonetheless, the migration of neural progenitors during development seem to involve complete channels between the neural progenitors and glial cells. GJ communication also plays a role in transmigration of lymphocytes during an immune response.

Studies by Oviedo-Orta et al show that calcein dye from the EC was transferred to the lymphocytes as they transmigrated, and that in the presence of a GJ communication inhibitor, this dye transfer was inhibited up to 60%. This also indicates that because migration was not completely inhibited, other factors may be involved. In 2007, Elias et al indicated that adhesion through Cx43 and Cx26 channels, and not the exchange of signaling molecules, are responsible for neuronal adhesion between contact points of radial fibres from neuronal stems cells, and is critical an enabling radial glial-guided neuronal migration. This discovery supports our findings that Cx43 GJ are involved in the adhesion and migration of cells as interrelated functions.

We hypothesized that as in the developing central nervous system, Cx43-containing GJ, expressed by both BMEC and HSC/P, could control the continuous HSC/P traffic through the sinusoidal BMEC in adult hematopoiesis, and given the in
vivo data, that the defect was only explained by a deficient retention of BM HSC/P. Our in vitro data support the data observed in vivo and an increased migration of HSC/P through Cx43-deficient BMEC.

Interestingly, a deficiency of Cx43 in HSC/P reverses the increased transendothelial migration through Cx43-deficient BMEC. These results suggest that the migration of HSC/P through BMEC does not depend on full Cx43 homomeric, homotypic channels, but does depend on Cx43 expression on HSC/P. We have not determined the levels of expression of Cx40 and Cx37 in BMEC are, therefore, it is possible that the deficiency of Cx43 in BMEC is compensated or even over-compensated, by normal or increased levels of Cx40 and Cx37, which are also expressed by BMEC.

To definitively demonstrate that the increased migration through Cx43-deficient BMEC corresponds to the in vivo phenotype of increased circulating HSC/P observed by Drs. Cancelas and Chang described earlier, an in vivo homing assay would be required. Previous data from our laboratory shows a 40% decrease in the homing of HSC/P to the BM microenvironment of osteoblast/stromal cell-specific (endosteal niche) Col-Cre Cx43 deficient mice compared to WT mice. Similarly, a homing assay of WT HSC/P into BMEC-specific Cx43-deficient mice would determine whether the adhesion/migration ratio is impaired. Our laboratory in vivo data along with the in vitro data reported in this thesis would suggest that a loss of retention of HSC/P in the marrow niches is secondary to Cx43 deficiency in BMEC. The modest adhesion defect observed in our in vitro studies would need to be confirmed in vivo by a homing assay.

Recently, Napoli et al demonstrated that parathyroid hormone (PTH) when administered in combination with G-CSF induces angiogenesis in a hind-limb
ischemia mouse model. PTH had been previously demonstrated to control the expansion of the osteoblast-dependent endosteal niche, and therefore the CXCL12 gradient between BM and PB. This treatment seems to mobilize and localize EC progenitors specifically to ischemic vascular cell beds and possibly modifies their proliferation to accelerate neoangiogenesis.

A large number of studies have indicated that Cx43 GJ, or the protein itself, is a tumor suppressor gene, and thereby a modulator of cell growth. It has been shown that many human tumor cell lines demonstrate deficient or aberrant loss of Cx expression, and that the exogenous expression of Cx43 can increase or restore cell growth control in both in vitro and in vivo studies. We hypothesized that the deficiency of Cx43 might impair EC proliferation, and therefore decrease the vessel content of the BM. Our analysis of Cx43 deficient and overexpressing BMEC shows a trend that supports our hypothesis. However, the lack of difference in the BMEC proliferation, compared with their control counterpart, makes us prudent in the conclusions obtained from these experiments. Further detailed experiments with other BMEC lines that have different levels of expression of Cx43, and preferably in the absence of SV40 transformation, are required to confirm these results.

We did not observe any change in the cell viability of Cx43-deficient BMEC. Cx43 expression has been associated with resistance to cell injury, and an increased cell death of Cx43-deficient BMEC would have been possible. Since no injury was applied to BMEC grown in vitro, we cannot rule out that under stress conditions, Cx43-deficiency would induce increased cell apoptosis/death. In any case, cell viability does not explain the decreased proliferation observed in Cx43 overexpressing BMEC.
In general, the differences in the proliferation, adhesion and transendothelial migration between WT and Cx43-deficient BMEC are modest. Possible pitfalls in this study include the fact that other Cx may play redundant or even compensatory roles on BMEC functions in relation with HSC/P, through the formation of heterotypic and/or heteromeric channels between HSC/P and BMEC such as Cx37 and Cx40. Indeed, it is known that Cx40 and Cx43 can form functional homomeric or heteromeric channels, which contribute to different functions on the EC. It is also possible that more than one mechanism involving these Cx is controlling the adhesion and migration of HSC/P. These possibilities cannot be ruled out without inhibiting the expression of Cx37 and Cx40. Another pitfall is that hematopoiesis is a complex process involving direct- and indirect- regulators. Besides BMEC, many other cytokines, growth factors and adhesion molecules involved in regulating HSC/P are released by other cells in the BM microenvironment. It is most likely that many mechanisms contribute to the adhesion and migration of HSC/P. The mechanistic role of BMEC Cx43 on hematopoiesis was therefore beyond the scope of this project.

Our results do suggest that Cx43 plays pleiotropic roles in the vascular stem cell niche. While the inhibition of Cx43 function in EC favors increased migration of HSC/P, the inhibition of Cx43 in HSC/P induces decreased migration.
SUMMARY

Our results support the following:

1. The overexpression of Cx43 seems to decrease BMEC proliferation, without affecting their cell viability.

2. The deficiency of Cx43 induced by RNA interference in BMEC lines induced decreased adhesion and increased migration of WT HSC/P.

3. The increased transendothelial migration induced by BMEC Cx43 deficiency is reverted by deficiency of Cx43 in HSC/P.

4. Cx43 seems to play distinct, cell-specific roles in the vascular niche.
REFERENCES


